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METHODS OF TREATING MULTIPLE  
MYELOMA AND MYELOMA-INDUCED BONE  
RESORPTION USING INTEGRIN ANTAGONISTS

5 This utility application is a continuation-in-  
part of United States application number 09/805,840, filed  
March 13, 2001, which is a continuation of PCT application  
number PCT/US99/21170, filed September 13, 1999, which  
claims priority of United States provisional application  
number 60/100,182, filed September 14, 1998. The  
10 disclosures of United States application number  
09/805,840, PCT application number PCT/US99/21170 and  
United States provisional application number 60/100,182  
are incorporated herein by reference.

TECHNICAL FIELD OF THE INVENTION

15 The present invention relates to a treatment for  
multiple myeloma, and the release of bone-resorbing  
factors by myeloma cells, resulting in severe bone loss,  
which is the major side-effect of myeloma in man. More  
particularly, this invention relates to integrin  
20 antagonists, such as antagonists of  $\alpha 4$  containing  
integrins, which inhibit the biological effects of such  
adhesion, associated with homing of multiple myeloma cells

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to bone marrow; their subsequent integrin- dependent survival; and their integrin-dependent release of bone-resorbing factors, resulting in bone destruction in patients with multiple myeloma.

5

BACKGROUND OF THE INVENTION

Multiple myeloma is a B-cell malignancy that has strong predilection for colonizing the bone marrow and is associated with severe osteoclastic bone resorption. Multiple myeloma is the second most common hematologic malignancy, with 15,000 new cases diagnosed each year and 30,000 to 40,000 myeloma patients in the U.S. annually (Mundy and Bertolini 1986). Eighty percent of the patients suffer from devastating osteolytic bone destruction caused by increased osteoclast (OCL) formation and activity (Mundy and Bertolini 1986). This bone destruction can cause excruciating bone pain, pathologic fractures, spinal cord compression, and life-threatening hypercalcemia. Because multiple myeloma cannot be cured by standard chemotherapy or stem cell transplantation (Attal et al., 1996), and because of the severe morbidity and potential mortality associated with myeloma bone disease, treatment strategies that control the myeloma growth itself, and in particular the osteolytic bone destruction that occurs in these patients, are vitally important.

However, the pathologic mechanisms responsible for the increased osteoclast activity in patients with multiple myeloma are unknown (Mundy, 1998). The bone lesions occur in several patterns. Occasionally, patients develop discrete osteolytic lesions that are associated

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with solitary plasmacytomas. Some patients have diffuse osteopenia, which mimics the appearance of osteoporosis, and is due to the myeloma cells being spread diffusely throughout the axial skeleton. In most patients there are multiple discrete lytic lesions occurring adjacent to nests of myeloma cells. Hypercalcemia occurs as a consequence of bone destruction in about one-third of patients with advanced disease. Rarely, patients with myeloma do not have lytic lesions or bone loss, but rather have an increase in the formation of new bone around myeloma cells. This rare situation is known as osteosclerotic myeloma.

Osteolytic bone lesions are by far the most common skeletal manifestations in patients with myeloma (Mundy, 1998). Although the precise molecular mechanisms remain unclear, observations over 15 years have shown that: 1) The mechanism by which bone is destroyed in myeloma is via the osteoclast, the normal bone-resorbing cell; 2) Osteoclasts accumulate on bone-resorbing surfaces in myeloma adjacent to collections of myeloma cells and it appears that the mechanism by which osteoclasts are stimulated in myeloma is a local one; 3) It has been known for many years that cultures of human myeloma cells in vitro produce several osteoclast activating factors; including lymphotoxin-alpha (LT- $\alpha$ ), interleukin-1(IL-1), parathyroid-hormone related protein (PTHrP) and interleukin-6 (IL-6); 4) Hypercalcemia occurs in approximately one-third of patients with myeloma some time during the course of the disease. Hypercalcemia is always associated with markedly increased bone resorption and frequently with impairment in glomerular filtration;

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5) The increase in osteoclastic bone resorption in myeloma is usually associated with a marked impairment in osteoblast function. Alkaline phosphatase activity in the serum is decreased or in the normal range, unlike patients with other types of osteolytic bone disease, and radionuclide scans do not show evidence of increased uptake, indicating impaired osteoblast responses to the increase in bone resorption.

Although various mediators listed above have been implicated in the stimulation of osteoclast activity in patients with multiple myeloma, reports of factors produced by myeloma cells have not been consistent, and some studies have been inconclusive due to the presence of other contaminating cell types, including stromal cells and macrophages, in the multiple myeloma cell population. IL-6 is a major myeloma growth factor that enhances the growth of several myeloma cell lines and freshly isolated myeloma cells from patients (Bataille et al., 1989). IL-6 production can be detected in about 40% of freshly isolated myeloma cells by PCR, but only 1 in 150 patients studied show detectable IL-6 production by immunocytochemistry or ELISA assays (Epstein 1992). The IL-6 receptors were only detected in 6 of 13 samples from patients with multiple myeloma (Bataille et al., 1992). Furthermore, mature myeloma cells have been reported to have a minimal proliferative response to IL-6. Interleukin-11 (IL-11) has an IL-6-like activity on plasmacytomas, but to date no one has demonstrated that myeloma cells produce IL-11. Bataille and coworkers (1995) have shown that perfusion of 5 patients with refractory myeloma with an antibody to IL-6 decreased the

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size of the myeloma cell burden in only 2 of these patients. IL-1 is an extremely potent bone resorbing agent that induces hypercalcemia in animal models in the absence of renal failure (Boyce et al., 1989). In contrast, hypercalcemia rarely occurs in myeloma patients without renal failure. More importantly, in highly purified myeloma cells, no IL-1 and only rare TNF- $\alpha$  production can be detected, suggesting that other contaminating cell types such as macrophages may be the source of IL-1 and TNF- $\alpha$  (Epstein 1992). Similarly, LT- $\alpha$  is produced by most human myeloma cell lines (Bataille et al., 1995) but does not appear to be produced by myeloma cells in vivo (Alsina et al., 1996). In addition to IL-1, TNF- $\alpha$ , LT- $\alpha$ , and IL-6, myeloma cells produce a truncated form of M-CSF which is biologically active, but M-CSF does not cause hypercalcemia or induce osteoclast formation by itself in human marrow cultures (MacDonald et al., 1986).

Thus, the role of any of these factors in osteolytic bone disease in patients with myeloma has not been clearly demonstrated in vivo, so that known cytokines clearly do not totally account for the bone resorption seen in these patients.

#### 25 Role of Adhesive Molecule Interactions in Myeloma Bone Disease

Anderson and coworkers were the first group to demonstrate the importance of adhesive interactions between myeloma cells and cells in the marrow microenvironment both in the growth of myeloma cells and the development of osteolytic bone disease. Multiple

myeloma cells express cell surface adhesion molecules, CD29 (VLA-4), LFA-1, and CD44 (Chauhan et al., 1995). These workers suggested that myeloma cells localized to the marrow via specific adhesion interactions between  
5 extracellular matrix proteins and bone marrow stromal cells. They further showed that adhesion of multiple myeloma cells to stromal cells triggered IL-6 secretion by both normal and multiple myeloma bone marrow-derived stromal cells and increased IL-6-mediated tumor cell  
10 growth. However, antibodies to CD29, LFA-1 or CD44 did not decrease IL-6 production by marrow stromal cells in response to myeloma cells, suggesting that another ligand-receptor interaction triggered the IL-6 secretion by bone marrow stromal cells binding to myeloma cells. Mere  
15 identification of a possible adhesion pathway does not necessarily mean that the pathway is important. In this case none of the implicated pathways plays a role in IL-6 production.

Vanderkerken et al. (1997) also examined the  
20 phenotypic adhesion profile of murine 5T2 cells and ST33 myeloma cells in a model of murine myeloma. These investigators showed that these cell lines expressed VLA-4, VLA-5, LFA-1, and CD44, and suggested that these adhesive interactions might be important for myeloma cells  
25 to bind to marrow stromal cells.

Nevertheless, despite many laboratory advances, the fundamental mechanisms underlying increased osteoclastic bone destruction in myeloma in vivo remain poorly understood. This is reflected in the inability to  
30 easily translate the data on adhesive interactions obtained in vitro to the in vivo setting. For example,

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many in vitro studies implicate both the integrin VLA-4 and the integrin LFA-1 in the adhesion of hematopoietic stem cells to bone marrow stroma (reviewed in Papayannopoulou and Nakamoto, 1993). These in vitro data  
5 would predict that either pathway, if blocked in vivo, would result in peripheralization of hematopoietic stem cells from marrow to peripheral blood. Yet, in a primate study, while a monoclonal antibody (mAb) to VLA-4 effectively peripheralized stem cells, a monoclonal  
10 antibody to the beta2 integrin chain of LFA-1 was without effect, despite increasing neutrophil counts, thus demonstrating the efficacy of the mAb (Papayannopoulou and Nakamoto, 1993). These data show that the in vitro results were in fact unable to accurately predict in vivo  
15 relevance.

It should be noted that the role of integrin VLA-4 has been studied in metastasis of multiple tumors, including leukemias such as lymphoma, with contradictory results. Thus, transfection of the human alpha 4 chain  
20 into Chinese Hamster Ovary (CHO) cells resulted in VLA-4 expression, and rendered these cells able to migrate to bone marrow in vivo, a phenomenon inhibited by mAbs to VLA-4 (Matsuura et al., 1996). In contrast, transfection of lymphoma cells with VLA-4 strongly inhibited metastasis  
25 to liver, lung and kidney, and was without effect on homing and proliferation in marrow (Gosslar et al., 1996). In addition, expression of VLA-4 on highly metastatic murine melanoma cells strongly inhibited the formation of pulmonary metastases in vivo (Qian et al., 1994), and did  
30 not predispose melanoma to bone marrow metastasis.

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In summary it is not clear on the basis of in vitro studies, how to reliably predict in vivo relevance of adhesion pathways. Furthermore, even when in vivo studies have been performed, the resultant data are  
5 inconsistent. One major reason for the perplexing inconsistencies in the field of multiple myeloma is that currently available animal models are not good predictors of human disease. In the case of multiple myeloma, human and murine myeloma cell lines which can be grown in vitro  
10 rarely are associated with bone destruction in vivo (Mundy 1998).

It would be highly desirable to identify compounds or antagonists which inhibit production of these bone-resorbing factors, thus halting progressive bone  
15 destruction and improving the quality of life of patients with myeloma.

#### SUMMARY OF THE INVENTION

We have used a recently developed murine model of multiple myeloma in which the mouse develops severe  
20 osteolysis with all the hallmarks of human disease (Garrett 1997). Using this cell line and animal model we have established that inhibition of the  $\alpha 4$  integrin/ $\alpha 4$  integrin ligand pathway in vivo leads to reduced capacity for multiple myeloma cells to proliferate and/or survive.  
25 We show that cell-cell attachment between myeloma cells and marrow stromal cells via the VLA-4/VCAM-1 interaction is required for an increase in the production of an activity which stimulates osteoclastic bone resorption in the bone microenvironment in vitro.

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We propose that this interaction is critical to the homing of myeloma cells to the marrow compartment, to their subsequent survival and growth, to ultimately to the progression of myeloma-induced osteolysis. We tested this  
5 in the animal model and found that, in vivo, an antagonist of the alpha4 subunit-containing integrin VLA-4 strongly inhibits the production of antibody of the IgG2b subtype. This isotype is the same as that produced by the 5TGM1 cell line, and is an accurate surrogate for the number of  
10 myeloma cells in the marrow compartment at any time. Thus, blockade of the VLA-4 pathway strongly inhibits IgG2b production, and by implication, the level of myeloma burden.

One aspect of the invention is a method for  
15 treating multiple myeloma comprising administering to an individual a therapeutically effective amount of a composition comprising an antagonist of an interaction between an integrin with an  $\alpha 4$  subunit (e.g., VLA-4) and a ligand for this integrin (e.g., VCAM-1). This  
20 antagonist can be an  $\alpha 4$  integrin binding agent or an  $\alpha 4$  integrin ligand binding agent. Preferred agents are anti-VLA4 or anti- $\alpha 4\beta 7$  antibody homologs (human antibody, a chimeric antibody, a humanized antibody and fragments thereof); anti-VCAM-1 antibody homologs (a human antibody;  
25 a chimeric antibody, a humanized antibody and fragments thereof); and a small molecule inhibitor of interactions of  $\alpha 4$  subunit containing integrins with their ligands. The composition can be administered at a dosage so as to provide from about 0.1. to about 20 mg/kg body weight. In  
30 particular, the preferred agents can antagonize an interaction: a) of both VLA-4 and  $\alpha 4\beta 7$  collectively with

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their respective  $\alpha 4$  ligands; or b) only of VLA-4 with its  $\alpha 4$  ligand; or c) only of  $\alpha 4\beta 7$  with its  $\alpha 4$  ligand. One or more antagonists of an interaction between an integrin with an  $\alpha 4$  subunit and a ligand for this integrin could  
5 be administered in combination with one or more compounds that are not an antagonist of an interaction between an integrin with an  $\alpha 4$  subunit and a ligand for this integrin. Preferably, the compound to be administered in combination with an antagonist of an interaction between  
10 an integrin with an  $\alpha 4$  subunit and a ligand for this integrin is a chemotherapeutic agent. Preferably, that chemotherapeutic agent is melphalan.

Another aspect of the invention is a method for inhibiting bone resorption associated with tumors of bone  
15 marrow, the method comprising administering to a mammal with said tumors an antagonist of an interaction between an  $\alpha 4$  subunit containing integrin such as VLA-4 and a ligand for this  $\alpha 4$  subunit containing integrin, such as VCAM-1, in an amount effective to provide inhibition of  
20 the bone resorption. This antagonist can be an  $\alpha 4$  integrin binding agent such as a VLA-4 binding agent or an  $\alpha 4$  integrin ligand binding agent such as a VCAM-1 binding agent. Preferred agents are anti-VLA4 or and  $\alpha 4\beta 7$  antibody homologs (human antibody, a chimeric antibody, a  
25 humanized antibody and fragments thereof); anti-VCAM-1 antibody homologs (a human antibody, a chimeric antibody, a humanized antibody and fragments thereof); and a small molecule inhibitor of the interaction of  $\alpha 4$  subunit-containing integrins with their respective  $\alpha 4$  integrin  
30 ligands (e.g, the VCAM-1/VLA-4 interaction). The antagonist can be administered at a dosage so as to

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provide from about 0.1 to about 20 mg/kg body weight. One or more antagonists of an interaction between an integrin with an  $\alpha 4$  subunit and a ligand for this integrin could be administered in combination with one or  
5 more compounds that are not an antagonist of an interaction between an integrin with an  $\alpha 4$  subunit and a ligand for this integrin. Preferably, the compound to be administered in combination with an antagonist of an interaction between an integrin with an  $\alpha 4$  subunit and a  
10 ligand for this integrin is a chemotherapeutic agent. Preferably, that chemotherapeutic agent is melphalan.

Yet another aspect of the invention is a method of treating a subject having a disorder characterized by the presence of osteoclastogenesis, the method comprising  
15 administering to the subject an antagonist of an interaction between an  $\alpha 4$  subunit bearing integrin and a ligand for an  $\alpha 4$  subunit-bearing integrin, in an amount sufficient to suppress the osteoclastogenesis. Similarly, the antagonist can be an  $\alpha 4$  binding agent or an  $\alpha 4$  ligand  
20 binding agent. Preferred agents are anti-VLA4 or anti- $\alpha 4\beta 7$  antibody homologs (human antibody, a chimeric antibody, a humanized antibody and fragments thereof); anti-VCAM-1 antibody homologs (a human antibody, a chimeric antibody, a humanized antibody and fragments  
25 thereof); and a small molecule inhibitor of the interaction of  $\alpha 4$  subunit-containing integrins with their respective  $\alpha 4$  integrin ligands (e.g, the VCAM-1/VLA-4 interaction). One or more antagonists of an interaction between an integrin with an  $\alpha 4$  subunit and a ligand for  
30 this integrin could be administered in combination with one or more compounds that are not an antagonist of an

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interaction between an integrin with an  $\alpha 4$  subunit and a ligand for this integrin. Preferably, the compound to be administered in combination with an antagonist of an interaction between an integrin with an  $\alpha 4$  subunit and a  
5 ligand for this integrin is a chemotherapeutic agent. Preferably, that chemotherapeutic agent is melphalan.

Unless stipulated otherwise, all references are incorporated herein by reference.

#### BRIEF DESCRIPTION OF THE FIGURES

10 The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Patent Office upon request and payment of the necessary fee.

15 **Figure 1.**

Effect of Neutralizing Antibodies on TRAP-positive Multinucleated OC-like Cell Formation in the  
Co-cultures of 5TGM1 cells and Bone Marrow Cells

A mixture of 5TGM1 cells (1 e 3) and marrow  
20 cells (1 e 6) in suspension was plated in 48-well culture plates and cultured with or without 10  $\mu\text{g/ml}$  anti-VCAM-1 antibody (VCAM-1 Ab), anti- $\alpha 4\beta 1$  antibody ( $\alpha 4\beta 1$ Ab), anti-ICAM-1 antibody (ICAM-1 Ab) or rat IgG as a control. After 6 days of culture, cultures were fixed and the  
25 number of TRAP-positive multinucleated OC-like cells (TRAP(+) MNC) determined. Both VCAM-1 Ab and  $\alpha 4\beta 1$  Ab inhibited TRAP(+) MNC formation, while ICAM-1 Ab had no effect. Data are expressed as mean  $\pm$  S.E. (n=3).

\* = Significantly different from IgG control.

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## Figure 2

### Effect of 5TGM1 and ST2 Conditioned Media on Bone Resorption in Organ Cultures of Fetal Rat Long Bones

Conditioned media (48 hours) obtained from ST2  
5 alone, 5TGM1 alone, and co-cultures of ST2 and 5TGM1 were  
assayed for bone resorbing activity in organ cultures of  
<sup>45</sup>calcium-labeled fetal rat long bones. Labeled fetal rat  
long bones were cultured in the presence of conditioned  
media (40%v/v) or control medium for 120 hours. Data are  
10 expressed as percentage increase of calcium release over  
than in the control medium. Release from conditioned  
medium of ST2 stromal cells is shown as the open bar.  
Release from 5TGM1 is the hatched bar. Release from  
conditioned medium harvested from co-culture of 5TGM1 and  
15 ST2 is the closed bar. Data are expressed as mean  $\pm$  S.E.  
(n=4). \* = significantly different from ST2 alone. \*\*\* =  
significantly different from 5TGM 1 alone.

## Figure 3

### Effect of Recombinant Soluble VCAM-1(sVCAM-1) on the Production of Osteoclastogenic Activity by 5TGM1 Cells

Conditioned medium was harvested from 5TGM1  
cells cultured in the presence or absence of sVCAM-1  
( $1 \times 10^{-8}$  to  $1 \times 10^{-7}$  Molar) for 24 hours. Osteoclastogenic  
activity of these conditioned media was assayed in the  
25 mouse marrow cultures. Bone marrow cells ( $1 \times 10^6$ /well) were  
plated into 48-well plates, and cultured in the presence  
of conditioned media (hatched bars) or control medium  
(IMDM) containing the same concentrations of sVCAM-1 (open  
bars). After 6 days, cultures were fixed and the number  
30 of TRAP-positive multinucleated OC-like cells (TRAP+MNC)  
was determined. Conditioned medium from 5TGM1 cells

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treated with  $1 \times 10^{-7}$  MsVCAM-1 increased TRAP(+)MNC formation. Data are expressed as mean  $\pm$  S.E. (n=3). \* = significantly different from controls.

#### Figure 4

5 Effect of mAb PS2 to VLA-4 on serum  
IgG2b elevation in 5TGM1-bearing mice

Mice were injected with  $1 \times 10^5$  5TGM1 cells, which were allowed to colonize the bone marrow. Mice were split into two groups of three, one serving as a control group, and the second treated on days 8, 11, 14, 17, and 20 with 80  $\mu$ g mAb PS/2 (~4 mg/kg). Levels of IgG2b, the antibody isotype produced by 5TGM1 myeloma cells, were measured weekly from weeks 1 to 6. mAb treatment strongly inhibited IgG2b production, indicative of inhibition of myeloma cell survival and growth in vivo.

#### Figure 5

Effect of mAb M/K-2.7 to VCAM-1 on  
serum IgG2b elevation in 5TGM1-bearing mice

Mice were injected with 5TGM1 cells as described in Figure 4, which were allowed to colonize the bone marrow. Mice were split into groups of four or five, one group serving as a control group (open square), the second/third groups treated prophylactically at 80  $\mu$ g (open diamonds) and 160  $\mu$ g mAb (open circles) (~4 to 8 mg/kg), the fourth group treated therapeutically at 160  $\mu$ g mAb (triangles). Levels of IgG2b, the antibody isotype produced by 5TGM1 myeloma cells, were measured. mAb treatment strongly inhibited IgG2b production, indicative of inhibition of myeloma cell survival and growth in vivo.

## Figure 6

## Effect of anti- $\alpha 4$ Integrin Antibody on Survival of Multiple Myeloma-bearing Mice

Figure 7 depicts an experimental protocol of an in vivo  
5 combination therapy experiment.

**Figure 8** shows the results (serum IgG2b levels in mice bearing 5TGM-1 cells) of an in vivo combination therapy experiment.

Figure 9 shows the results (tumor volume in bone) of an in vivo combination therapy experiment.

**Figure 10** shows the results (spleen weights) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent Raj mice.

15 **Figure 11** shows the results (percentage of myeloma cells in the spleen) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immunocompetent Raj mice.

20 **Figure 12** shows the representative FACS plots of the staining for lineage markers and cytoplasmic mouse IgG2b in splenocytes of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-  
25 competent Raj mice.



**Figure 13** shows the results (spleen tumor burden) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent Raj mice.

- 5 **Figure 14** shows the results (effects on bone marrow cells) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent Raj mice.

- 10 **Figure 15** shows the results (bone marrow tumor burden) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent Raj mice.

- 15 **Figure 16** shows the results (effects on 5TGM1 myeloma cells in peripheral blood) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent Raj mice.

- 20 **Figure 17** shows the results (effects on blood chemistry) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent Raj mice.

- 25 **Figure 18** shows the results (effects on circulating mIgG2b levels at the conclusion of the experiment) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent Raj mice.

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**Figure 19** depicts an experimental acute treatment protocol of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent Raj mice.

5 **Figure 20** shows the results (effects on IgG2b positive cells; 1 and 2 day treatments) of an acute treatment protocol of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-  
10 competent Raj mice.

**Figure 21** shows the results (effects on tumor burden in spleen; 1 and 2 day treatments) of an acute treatment protocol of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple  
15 myeloma. The mice of this model system were immuno-competent Raj mice.

**Figure 22** shows the results (effects on IgG2b positive cells; 6 day treatments) of an acute treatment protocol of an experiment relating to PS/2 mAb administration in an in  
20 vivo model system for multiple myeloma. The mice of this model system were immuno-competent Raj mice.

**Figure 23** shows the results (effects on tumor burden in bone marrow; 6 day treatments) of an acute treatment protocol of an experiment relating to PS/2 mAb  
25 administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent Raj mice.

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**Figure 24** depicts an experimental protocol of an in vivo combination therapy experiment in immunocompetent Raj mice.

**Figure 25** shows the results that melphalan and PS/2 mAb together, but not either alone, reduced mIgG2b levels in an in vivo combination therapy experiment in immunocompetent Raj mice.

**Figure 26** shows the results (effects of treatment on myeloma cells at day 27) of an in vivo combination therapy experiment in immunocompetent Raj mice.

**Figure 27** shows the results (effects of treatment on tumor burden at day 27) of an in vivo combination therapy experiment in immunocompetent Raj mice.

**Figure 28** shows PS/2 mAb plasma levels of an in vivo combination therapy experiment in immunocompetent Raj mice.

**Figure 29** depicts an experimental protocol of an experiment relating to BIO8809 administration in an in vivo model system for multiple myeloma.

**Figure 30** shows the results (effects of long term treatment on myeloma cells) of an experiment relating to BIO8809 administration in an in vivo model system for multiple myeloma.

**Figure 31** shows the results (effects of long term treatment on tumor burden) of an experiment relating to

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BIO8809 administration in an in vivo model system for multiple myeloma.

**Figure 32** depicts an acute treatment protocol of an experiment relating to BIO8809 administration in an in vivo model system for multiple myeloma.

**Figure 33** shows the results (effects of acute treatment on myeloma cells) of an experiment relating to BIO8809 administration in an in vivo model system for multiple myeloma.

**Figure 34** shows the results (effects of acute term treatment on tumor burden) of an experiment relating to BIO8809 administration in an in vivo model system for multiple myeloma.

**Figure 35** depicts an experimental protocol of an in vivo combination therapy experiment relating to survival as measured by the onset of paralysis.

**Figure 36** shows the results fo an in vivo combination therapy experiment relating to survival as measured by the onset of paralysis.

**DETAILED DESCRIPTION OF THE INVENTION**

The invention relates to treatments for, among other things, preventing multiple myeloma. More particularly, methods of the invention relate to the use of antagonists of an interaction between an integrin containing an  $\alpha 4$  subunit and a ligand for this integrin

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in the treatment of multiple myeloma. The term "multiple myeloma" is intended to mean a medical condition in an individual having a neoplastic disease of plasma cells, with the neoplastic clone representing cells at different stages in the plasma cell lineage from patient to patient (Mundy, 1998).

Alpha 4  $\beta$ 1 integrin is a cell-surface receptor for VCAM-1, fibronectin and possibly other molecules that bind with, or otherwise interact with, alpha 4  $\beta$ 1 integrin. In this regard, such molecules that bind with, or otherwise interact with, alpha 4 subunit containing integrin are individually and collectively referred to as " $\alpha$ 4 ligand(s)"). The term  $\alpha$ 4 $\beta$ 1 integrin ("VLA-4" or " $\alpha$ 4 $\beta$ 1, or " $\alpha$ 4 $\beta$ 1 integrin", used interchangeably) herein thus refers to polypeptides which are capable of binding to VCAM-1 and members of the extracellular matrix proteins, most particularly fibronectin, or homologs or fragments thereof, although it will be appreciated by workers of ordinary skill in the art that other ligands for VLA-4 may exist and can be analyzed using conventional methods.

Nevertheless, it is known that the  $\alpha$ 4 subunit will associate with other  $\beta$  subunits besides  $\beta$ 1 so that we may define the term " $\alpha$ 4 integrin" as being those integrins whose  $\alpha$ 4 subunit associates with one or another of the  $\beta$  subunits. A further example of an " $\alpha$ 4" integrin is  $\alpha$ 4 $\beta$ 7 (R. Lobb and M. Hemler, 1994). As used herein, the term " $\alpha$ 4 integrin(s)" means VLA-4, as well as integrins that contain  $\beta$ 1,  $\beta$ 7 or any other  $\beta$  subunit.

As discussed herein, the antagonists used in methods of the invention are not limited to a particular

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type or structure of molecule so that, for purposes of the invention, any agent capable of binding to any integrin containing an  $\alpha 4$  subunit such as VLA-4 on the surface of VLA-4 bearing cells and/or  $\alpha 4\beta 7$  integrin on the surface of  $\alpha 4\beta 7$ -bearing cells [see Lobb and Hemler, J. Clin. Invest., 94: 1722-1728 (1994)] and/or to their respective  $\alpha 4$  ligands such as VCAM-1 and MadCAM, respectively, on the surface of VCAM-1 and MadCAM bearing cells, and which effectively blocks or coats VLA-4 (or  $\alpha 4\beta 7$ ) or VCAM-1 (or MadCAM) (i.e., a "an  $\alpha 4$  integrin binding agent" and " $\alpha 4$  integrin ligand binding agent" respectively), is considered to be an equivalent of the antagonists used in the examples herein.

An integrin "antagonist" includes any compound that inhibits an  $\alpha 4$  integrin(s) from binding with an  $\alpha 4$  integrin ligand and/or receptor. Anti-integrin antibody or antibody homolog-containing proteins (discussed below) as well as other molecules such as soluble forms of the ligand proteins for integrins are useful. Soluble forms of the ligand proteins for  $\alpha 4$  integrins include soluble VCAM-1 or collagen peptides, VCAM-1 fusion protein, or bifunctional VCAM-1/Ig fusion proteins. For example, a soluble form of an  $\alpha 4$  integrin ligand or a fragment thereof may be administered to bind to integrin, and preferably compete for an integrin binding site on cells, thereby leading to effects similar to the administration of antagonists such as anti- $\alpha 4$  integrin (e.g.,  $\alpha 4\beta 7$  antibodies and/or VLA-4 antibodies). In particular, soluble  $\alpha 4$  integrin mutants that bind  $\alpha 4$  integrin ligand but do not elicit integrin-dependent signaling are included within the scope of the invention. Such mutants

can act as competitive inhibitors of wild type integrin protein and are considered "antagonists". Other antagonists used in the methods of the invention are "small molecules", as defined below.

- 5           Included within the invention are methods using an agent that antagonizes the action of more than one  $\alpha 4$  integrin, such as a single small molecule or antibody homolog that antagonizes several  $\alpha 4$  integrins such as VLA-4 and  $\alpha 4\beta 7$ , or other combinations of  $\alpha 4$  integrins.
- 10   Also included within the scope of the invention are methods using a combination of different molecules such that the combined activity antagonizes the action of more than one  $\alpha 4$  integrin, such as methods using several small molecules or antibody homologs that in combination
- 15   antagonize the  $\alpha 4$  integrins VLA-4 and  $\alpha 4\beta 7$ , or other combinations of integrins.

- As discussed herein, certain integrin antagonists can be fused or otherwise conjugated to, for instance, an antibody homolog such as an immunoglobulin or
- 20   fragment thereof and are not limited to a particular type or structure of an integrin or ligand or other molecule. Thus, for purposes of the invention, any agent capable of forming a fusion protein (as defined below) and capable of binding to  $\alpha 4$  integrin ligands and which effectively
- 25   blocks or coats  $\alpha 4\beta 7$  and/or VLA-4 integrin is considered to be an equivalent of the antagonists used in the examples herein.

- For the purposes of the invention an "antagonist of the  $\alpha 4$  integrin ligand/ $\alpha 4$  integrin interaction" refers
- 30   to an agent, e.g., a polypeptide or other molecule, which can inhibit or block  $\alpha 4$  ligand (e.g., VCAM-1) and/or  $\alpha 4$

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integrin (e.g.,  $\alpha 4\beta 7$  or VLA-4)-mediated binding or which can otherwise modulate  $\alpha 4$  ligand and/or  $\alpha 4$  integrin function, e.g., by inhibiting or blocking  $\alpha 4$ -ligand-mediated  $\alpha 4$  integrin signal transduction or  $\alpha 4$  ligand mediated  $\alpha 4$  ligand signal transduction and which is effective in the treatment of multiple myeloma, preferably in the same manner as are anti- $\alpha 4$  integrin antibodies.

Specifically, an antagonist of the VCAM-1/VLA-4 interaction is an agent which has one or more of the following properties: (1) it coats, or binds to VLA-4 on the surface of a VLA-4 bearing cell (e.g., a myeloma cell) with sufficient specificity to inhibit a VLA-4-ligand/VLA-4 interaction, e.g., the VCAM-1/VLA-4 interaction between bone stromal cells and myeloma cells; (2) it coats, or binds to, VLA-4 on the surface of a VLA-4 bearing cell (i.e., a myeloma cell) with sufficient specificity to modify, and preferably to inhibit, transduction of a VLA-4-mediated signal e.g., VLA-4/VCAM-1-mediated signaling; (3) it coats, or binds to, a VLA-4-ligand, (e.g., VCAM-1) on bone stromal cells with sufficient specificity to inhibit the VLA-4/VCAM interaction; (4) it coats, or binds to, a VLA-4-ligand (e.g., VCAM-1) on bone stromal cells with sufficient specificity to modify, and preferably to inhibit, transduction of VLA-4-ligand mediated VLA-4 signaling, e.g., VCAM-1-mediated VLA-4 signaling. In preferred embodiments the antagonist has one or both of properties 1 and 2. In other preferred embodiments the antagonist has one or both of properties 3 and 4. Moreover, more than one antagonist can be administered to a patient, e.g., an

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agent which binds to VLA-4 can be combined with an agent which binds to VCAM-1.

For example, antibodies or antibody homologs (discussed below) as well as soluble forms of the natural binding proteins for VLA-4 and VCAM-1 are useful. Soluble forms of the natural binding proteins for VLA-4 include soluble VCAM-1 peptides, VCAM-1 fusion proteins, bifunctional VCAM-1/Ig fusion proteins, fibronectin, fibronectin having an alternatively spliced non-type III connecting segment, and fibronectin peptides containing the amino acid sequence EILDV or a similar conservatively substituted amino acid sequence. Soluble forms of the natural binding proteins for VCAM-1 include soluble VLA-4 peptides, VLA-4 fusion proteins, bifunctional VLA-4/Ig fusion proteins and the like. As used herein, a "soluble VLA-4 peptide" or a "soluble VCAM-1 peptide" is an VLA-4 or VCAM-1 polypeptide incapable of anchoring itself in a membrane. Such soluble polypeptides include, for example, VLA-4 and VCAM polypeptides that lack a sufficient portion of their membrane spanning domain to anchor the polypeptide or are modified such that the membrane spanning domain is non-functional. These binding agents can act by competing with the cell-surface binding protein for VLA-4 or by otherwise altering VLA-4 function. For example, a soluble form of VCAM-1 (see, e.g., Osborn et al. 1989, Cell, 59: 1203-1211) or a fragment thereof may be administered to bind to VLA-4, and preferably compete for a VLA-4 binding site on myeloma cells, thereby leading to effects similar to the administration of antagonists such as small molecules or anti-VLA-4 antibodies.

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In another example, VCAM-1, or a fragment thereof, which is capable of binding to VLA-4 on the surface of VLA-4 bearing myeloma cells, e.g., a fragment containing the two N-terminal domains of VCAM-1, can be  
5 fused to a second peptide, e.g., a peptide which increases the solubility or the in vivo life time of the VCAM-1 moiety. The second peptide can be a fragment of a soluble peptide, preferably a human peptide, more preferably a plasma protein, or a member of the immunoglobulin  
10 superfamily. In particularly preferred embodiments the second peptide is IgG or a portion or fragment thereof, e.g., the human IgG1 heavy chain constant region and includes, at least the hinge, CH2 and CH3 domains.

Other antagonists useful in the methods of the  
15 invention include, but are not limited to, agents that mimic the action of peptides (organic molecules called "small molecules") capable of disrupting the  $\alpha 4$  integrin/ $\alpha 4$  integrin ligand interaction by, for instance, blocking VLA-4 by binding VLA-4 receptors on the surface  
20 of cells or blocking VCAM-1 by binding VCAM-1 receptors on the surface of cells. These "small molecules" may themselves be small peptides, or larger peptide-containing organic compounds or non-peptidic organic, compounds. A "small molecule", as defined herein, is not intended to  
25 encompass an antibody or antibody homolog. Although the molecular weight of such "small molecules" is generally less than 2000, we don't intend to apply this figure as an absolute upper limit on molecular weight.

For instance, small molecules such as  
30 oligosaccharides that mimic the binding domain of a VLA-4 ligand and fit the receptor domain of VLA-4 may be

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5 molecules that mimic the binding domain of a VCAM-1 ligand and fit the receptor domain of VCAM-1 may be employed.

10 Site (CS1) Within the Alternatively Spliced Type III  
Connecting Segment Domain of Fibronectin Is  
Leucine-Aspartic Acid-Valine", J. Biol. Chem., 266 (23),  
pp. 15075-79 (1991)). They identified the minimum active  
amino acid sequence necessary to bind VLA-4 and  
15 synthesized a variety of overlapping peptides based on the  
amino acid sequence of the CS-1 region (the VLA-4 binding  
domain) of a particular species of fibronectin. They  
identified an 8-amino acid peptide, Glu-Ile-Leu-Asp-Val-  
Pro-Ser-Thr, as well as two smaller overlapping  
20 pentapeptides, Glu-Ile-Leu-Asp-Val and  
Leu-Asp-Val-Pro-Ser, that possessed inhibitory activity  
against fibronectin-dependent cell adhesion. Certain  
larger peptides containing the LDV sequence were  
subsequently shown to be active in vivo (T.A. Ferguson  
25 et al., "Two Integrin Binding Peptides Abrogate  
T-cell-Mediated Immune Responses In Vivo", Proc. Natl.  
Acad. Sci. USA, 88, pp. 8072-76 (1991); and S. M. Wahl  
et al., "Synthetic Fibronectin Peptides Suppress Arthritis  
in Rats by Interrupting Leukocyte Adhesion and  
30 Recruitment", J. Clin. Invest., 94, pp. 655-62 (1994)).  
A cyclic pentapeptide, Arg-Cys-Asp-TPro-Cys (wherein TPro

denotes 4-thioprolino), which can inhibit both VLA-4 and VLA-5 adhesion to fibronectin has also been described.

(See, e.g., D.M. Nowlin et al., "A Novel Cyclic Pentapeptide Inhibits Alpha4Beta1 Integrin-mediated Cell Adhesion", J. Biol. Chem., 268(27), pp. 20352-59 (1993); and PCT publication PCT/US91/04862). This pentapeptide was based on the tripeptide sequence Arg-Gly-Asp from FN which had been known as a common motif in the recognition site for several extracellular-matrix proteins.

10           Examples of other small molecule VLA-4 inhibitors have been reported, for example, in Adams et al., "Cell Adhesion Inhibitors", PCT US97/13013, describing linear peptidyl compounds containing beta-amino acids which have cell adhesion inhibitory activity.

15           International patent applications WO 94/15958 and WO 92/00995 describe cyclic peptide and peptidomimetic compounds with cell adhesion inhibitory activity. International patent applications WO 93/08823 and WO 92/08464 describe guanidiny-, urea- and

20           thiourea-containing cell adhesion inhibitory compounds. United States Patent No. 5,260,277 describes guanidiny cell adhesion modulation compounds.

          Yet other examples of small molecules that bind to or otherwise interact with VLA-4 molecules and inhibit

25           VLA-4 dependent cell adhesion are disclosed in WO 01/12186, the disclosure of which is incorporated by reference herein.

          Such small molecules mimetic agents may be produced by synthesizing a plurality of peptides

30           semi-peptidic compounds or non-peptidic, organic compounds, and then screening those compounds for their

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ability to inhibit the  $\alpha 4$  integrin/ $\alpha 4$  integrin ligand interaction. See generally U.S. Patent No. 4,833,092, Scott and Smith, "Searching for Peptide Ligands with an Epitope Library", Science, 249, pp. 386-90 (1990), and  
5 Devlin et al., "Random Peptide Libraries: A Source of Specific Protein Binding Molecules", Science, 249, pp. 40407 (1990).

In other preferred embodiments, the agent that is used in the method of the invention to bind to,  
10 including block or coat, cell-surface  $\alpha 4$  integrin and/or  $\alpha 4$  integrin ligand is an anti-VLA-4 and/or anti- $\alpha 4\beta 7$  monoclonal antibody or antibody homolog. Preferred antibodies and homologs for treatment, in particular for human treatment, include human antibody homologs,  
15 humanized antibody homologs, chimeric antibody homologs, Fab, Fab', F(ab')<sub>2</sub> and F(v) antibody fragments, and monomers or dimers of antibody heavy or light chains or mixtures thereof. Monoclonal antibodies against VLA-4 are a preferred binding agent in the method of the invention.

20 As used herein, the term "antibody homolog" includes intact antibodies consisting of immunoglobulin light and heavy chains linked via disulfide bonds. The term "antibody homolog" is also intended to encompass a protein comprising one or more polypeptides selected from  
25 immunoglobulin light chains, immunoglobulin heavy chains and antigen-binding fragments thereof which are capable of binding to one or more antigens. The component polypeptides of an antibody homolog composed of more than one polypeptide may optionally be disulfide-bound or  
30 otherwise covalently crosslinked.

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Accordingly, therefore, "antibody homologs" include intact immunoglobulins of types IgA, IgG, IgE, IgD, IgM (as well as subtypes thereof), wherein the light chains of the immunoglobulin may be of types kappa or  
5 lambda.

"Antibody homologs" also include portions of intact antibodies that retain antigen-binding specificity, for example, Fab fragments, Fab' fragments, F(ab')<sub>2</sub> fragments, F(v) fragments, heavy chain monomers or dimers,  
10 light chain monomers or dimers, dimers consisting of one heavy and one light chain, and the like. Thus, antigen-binding fragments, as well as full-length dimeric or trimeric polypeptides derived from the above-described antibodies are themselves useful.

As used herein, a "humanized antibody homolog" is an antibody homolog, produced by recombinant DNA technology, in which some or all of the amino acids of a human immunoglobulin light or heavy chain that are not required for antigen binding have been substituted for the  
15 corresponding amino acids from a nonhuman mammalian immunoglobulin light or heavy chain.

As used herein, a "chimeric antibody homolog" is an antibody homolog, produced by recombinant DNA technology, in which all or part of the hinge and constant  
25 regions of an immunoglobulin light chain, heavy chain, or both, have been substituted for the corresponding regions from another immunoglobulin light chain or heavy chain. In another aspect the invention features a variant of a chimeric molecule which includes: (1) a VLA-4 targeting moiety, e.g., a VCAM-1 moiety capable of binding to  
30 antigen (i.e., VLA-4) on the surface of VLA-4 bearing

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myeloma cells; (2) optionally, a second peptide, e.g., one which increases solubility or in vivo life time of the VLA-4 targeting moiety, e.g., a member of the immunoglobulin superfamily or fragment or portion thereof, e.g., a portion or a fragment of IgG, e.g., the human IgG1 heavy chain constant region, e.g., CH2 and CH3 hinge regions; and a toxin moiety. The VLA-4 targeting moiety can be any naturally occurring VLA-4 ligand or fragment thereof, e.g., a VCAM-1 peptide or a similar conservatively substituted amino acid sequence. A preferred targeting moiety is a soluble VCAM-1 fragment, e.g., the N-terminal domains 1 and 2 of the VCAM-1 molecule. The chimeric molecule can be used to treat a subject, e.g., a human, at risk for disorder, e.g., multiple myeloma, characterized by the presence of myeloma cells bearing VLA-4, and preferably activated VLA-4.

As used herein, a "human antibody homolog" is an antibody homolog produced by recombinant DNA technology, in which all of the amino acids of an immunoglobulin light or heavy chain that are derived from a human source.

#### Methods of Making Anti-VLA-4 Antibody Homologs

The technology for producing monoclonal antibody homologs is well known. Briefly, an immortal cell line (typically myeloma cells) is fused to lymphocytes (typically splenocytes) from a mammal immunized with whole cells expressing a given antigen, e.g., VLA-4, and the culture supernatants of the resulting hybridoma cells are screened for antibodies against the antigen. See, generally, Kohler et al., 1975, Nature, 265: 295-297.

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Immunization may be accomplished using standard procedures. The unit dose and immunization regimen depend on the species of mammal immunized, its immune status, the body weight of the mammal, etc. Typically, the immunized mammals are bled and the serum from each blood sample is assayed for particular antibodies using appropriate screening assays. For example, anti-VLA-4 antibodies may be identified by immunoprecipitation of <sup>125</sup>I-labeled cell lysates from VLA-4-expressing cells. (See, Sanchez-Madrid et al., 1986, Eur. J. Immunol., 16: 1343-1349 and Hemler et al., 1987, J. Biol. Chem., 262, 11478-11485). Anti-VLA-4 antibodies may also be identified by flow cytometry, e.g., by measuring fluorescent staining of Ramos cells incubated with an antibody believed to recognize VLA-4 (see, Elices et al., 1990 Cell, 60: 577-584). The lymphocytes used in the production of hybridoma cells typically are isolated from immunized mammals whose sera have already tested positive for the presence of anti-VLA-4 antibodies using such screening assays.

Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using 1500 molecular weight polyethylene glycol ("PEG 1500"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days



because they are not transformed). Hybridomas producing a desired antibody are detected by screening the hybridoma culture supernatants. For example, hybridomas prepared to produce anti-VLA-4 antibodies may be screened by testing  
5 the hybridoma culture supernatant for secreted antibodies having the ability to bind to a recombinant  $\alpha 4$ -subunit-expressing cell line (see, Elices et al., supra).

To produce anti-VLA-4 antibody homologs that are intact immunoglobulins, hybridoma cells that tested  
10 positive in such screening assays were cultured in a nutrient medium under conditions and for a time sufficient to allow the hybridoma cells to secrete the monoclonal antibodies into the culture medium. Tissue culture techniques and culture media suitable for hybridoma cells  
15 are well known. The conditioned hybridoma culture supernatant may be collected and the anti-VLA4 antibodies optionally further purified by well-known methods.

Alternatively, the desired antibody may be produced by injecting the hybridoma cells into the  
20 peritoneal cavity of an unimmunized mouse. The hybridoma cells proliferate in the peritoneal cavity, secreting the antibody which accumulates as ascites fluid. The antibody may be harvested by withdrawing the ascites fluid from the peritoneal cavity with a syringe.

25 Several mouse anti-VLA-4 monoclonal antibodies have been previously described. See, e.g., Sanchez-Madrid et al., 1986, supra; Hemler et al., 1987, supra; Pulido et al., 1991, J. Biol. Chem., 266 (16), 10241-10245). These anti-VLA-4 monoclonal antibodies such as HP ½ and  
30 other anti-VLA-4 antibodies (e.g., HP2/1, HP2/4, L25, P4C2, P4G9) capable of recognizing the P chain of VLA-4

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will be useful in the methods of treatment according to the present invention. Anti VLA-4 antibodies that will recognize the VLA-4  $\alpha 4$  chain epitopes involved in binding to VCAM-1 and fibronectin ligands (i.e., antibodies which  
5 can bind to VLA-4 at a site involved in ligand recognition and block VCAM-1 and fibronectin binding) are preferred. Such antibodies have been defined as B epitope-specific antibodies (B1 or B2) (Pulido et al., 1991, supra) and are also anti-VLA-4 antibodies according to the present  
10 invention.

Fully human monoclonal antibody homologs against VLA-4 are another preferred binding agent which may block or coat VLA-4 antigens in the method of the invention. In their intact form these may be prepared using in  
15 vitro-primed human splenocytes, as described by Boerner et al., 1991, J. Immunol., 147, 86-95. Alternatively, they may be prepared by repertoire cloning as described by Persson et al., 1991, Proc. Nat. Acad. Sci. USA, 88: 2432-2436 or by Huang and Stollar, 1991, J. Immunol.  
20 Methods 141, 227-236. U.S. Patent 5,798,230 (Aug. 25, 1998, "Process for the preparation of human monoclonal antibodies and their use") who describe preparation of human monoclonal antibodies from human B cells. According to this process, human  
25 antibody-producing B cells are immortalized by infection with an Epstein-Barr virus, or a derivative thereof, that expresses Epstein-Barr virus nuclear antigen 2 (EBNA2). EBNA2 function, which is required for immortalization, is subsequently shut off, which results in an increase in  
30 antibody production.

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In yet another method for producing fully human antibodies, United States Patent 5,789,650 (Aug. 4, 1998, "Transgenic non-human animals for producing heterologous antibodies") describes transgenic non-human animals

5 capable of producing heterologous antibodies and transgenic non-human animals having inactivated endogenous immunoglobulin genes. Endogenous immunoglobulin genes are suppressed by antisense polynucleotides and/or by antiserum directed against endogenous immunoglobulins.

10 Heterologous antibodies are encoded by immunoglobulin genes not normally found in the genome of that species of non-human animal. One or more transgenes containing sequences of unrearranged heterologous human immunoglobulin heavy chains are introduced into a

15 non-human animal thereby forming a transgenic animal capable of functionally rearranging transgenic immunoglobulin sequences and producing a repertoire of antibodies of various isotypes encoded by human immunoglobulin genes. Such heterologous human antibodies

20 are produced in B-cells which are thereafter immortalized, e.g., by fusing with an immortalizing cell line such as a myeloma or by manipulating such B-cells by other techniques to perpetuate a cell line capable of producing a monoclonal heterologous, fully human antibody homolog.

25 Large nonimmunized human phage display libraries may also be used to isolate high affinity antibodies that can be developed as human therapeutics using standard phage technology (Vaughan et al., 1996). Yet another preferred binding agent which may block or coat VLA-4

30 antigens in the method of the invention is a humanized recombinant antibody homolog having anti-VLA-4

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specificity. Following the early methods for the preparation of chimeric antibodies, a new approach was described in EP 0239400 (Winter et al.) whereby antibodies are altered by substitution of their complementarity determining regions (CDRs) for one species with those from another. This process may be used, for example, to substitute the CDRs from human heavy and light chain Ig variable region domains with alternative CDRs from murine variable region domains. These altered Ig variable regions may subsequently be combined with human Ig constant regions to create antibodies which are totally human in composition except for the substituted murine CDRs. Such CDR-substituted antibodies would be predicted to be less likely to elicit an immune response in humans compared to chimeric antibodies because the CDR-substituted antibodies contain considerably less non-human components. The process for humanizing monoclonal antibodies via CDR "grafting" has been termed "reshaping". (Riechmann et al., 1988, Nature 332, 323-327; Verhoeyen et al., 1988, Science 239, 1534-1536).

Typically, complementarity determining regions (CDRs) of a murine antibody are transplanted onto the corresponding regions in a human antibody, since it is the CDRs (three in antibody heavy chains, three in light chains) that are the regions of the mouse antibody which bind to a specific antigen. Transplantation of CDRs is achieved by genetic engineering whereby CDR DNA sequences are determined by cloning of murine heavy and light chain variable (V) region gene segments, and are then transferred to corresponding human V regions by site directed mutagenesis. In the final stage of the process,

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human constant region gene segments of the desired isotype (usually gamma I for CH and kappa for CL) are added and the humanized heavy and light chain genes are co-expressed in mammalian cells to produce soluble humanized antibody.

5           The transfer of these CDRs to a human antibody confers on this antibody the antigen binding properties of the original murine antibody. The six CDRs in the murine antibody are mounted structurally on a V region "framework" region. The reason that CDR-grafting is  
10 successful is that framework regions between mouse and human antibodies may have very similar 3-D structures with similar points of attachment for CDRs, such that CDRs can be interchanged. Such humanized antibody homologs may be prepared, as exemplified in Jones et al., 1986, Nature  
15 321, 522-525; Riechmann, 1988, Nature 332, 323-327; Queen et al., 1989, Proc. Nat. Acad. Sci. USA 86, 10029; and Orlandi et al., 1989, Proc. Nat. Acad. Sci. USA 86, 3833.

Nonetheless, certain amino acids within framework regions are thought to interact with CDRs and to  
20 influence overall antigen binding affinity. The direct transfer of CDRs from a murine antibody to produce a recombinant humanized antibody without any modifications of the human V region frameworks often results in a partial or complete loss of binding affinity. In a number  
25 of cases, it appears to be critical to alter residues in the framework regions of the acceptor antibody in order to obtain binding activity.

Queen et al., 1989 (supra) and WO 90/07861 (Protein Design Labs) have described the preparation of a  
30 humanized antibody that contains modified residues in the framework regions of the acceptor antibody by combining

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the CDRs of a murine mAb (anti-Tac) with human immunoglobulin framework and constant regions. They have demonstrated one solution to the problem of the loss of binding affinity that often results from direct CDR transfer without any modifications of the human V region framework residues; their solution involves two key steps. First, the human V framework regions are chosen by computer analysts for optimal protein sequence homology to the V region framework of the original murine antibody, in this case, the anti-Tac mAb. In the second step, the tertiary structure of the murine V region is modeled by computer in order to visualize framework amino acid residues which are likely to interact with the murine CDRs and these murine amino acid residues are then superimposed on the homologous human framework. See also Protein Design Labs -- U.S. Patent 5,693,762.

One may use a different approach (Tempest et al., 1991, Biotechnology 9, 266-271) and utilize, as standard, the V region frameworks derived from NEWM and REI heavy and light chains respectively for CDR-grafting without radical introduction of mouse residues. An advantage of using the Tempest et al., approach to construct NEWM and REI based humanized antibodies is that the 3-dimensional structures of NEWM and REI variable regions are known from x-ray crystallography and thus specific interactions between CDRs and V region framework residues can be modeled.

Regardless of the approach taken, the examples of the initial humanized antibody homologs prepared to date have shown that it is not a straightforward process. However, even acknowledging that such framework changes

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may be necessary, it is not possible to predict, on the basis of the available prior art, which, if any, framework residues will need to be altered to obtain functional humanized recombinant antibodies of the desired  
5 specificity. Results thus far indicate that changes necessary to preserve specificity and/or affinity are for the most part unique to a given antibody and cannot be predicted based on the humanization of a different antibody.

10 Preferred antagonists useful in the present invention include chimeric recombinant and humanized recombinant antibody homologs (i.e., intact immunoglobulins and portions thereof) with B epitope specificity that have been prepared and are described in  
15 co-pending U.S. Patent Application Serial No. 08/004,798, filed January 12, 1993, PCT Publication US94/00266, filed January 7, 1994. The starting material for the preparation of chimeric (mouse V - human C) and humanized anti-VLA-4 antibody homologs may be a murine monoclonal  
20 anti-VLA-4 antibody as previously described, a monoclonal anti-VLA-4 antibody commercially available (e.g., HP2/1, Amac International, Inc., Westbrook, Maine), or a monoclonal anti-VLA-4 antibody prepared in accordance with the teaching herein. For example, the variable regions of  
25 the heavy and light chains of the anti-VLA-4 antibody HP ½ have been cloned, sequenced and expressed in combination with constant regions of human immunoglobulin heavy and light chains. Such HP ½ antibody is similar in specificity and potency to the murine HP ½ antibody, and  
30 may be useful in methods of treatment according to the present invention.

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Other preferred humanized anti-VLA4 antibody homologs are described by Athena Neurosciences, Inc. in PCT/US95/01219 (27 July 1995). These humanized anti-VLA-4 antibodies comprise a humanized light chain and a humanized heavy chain. The humanized light chain comprises three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the corresponding complementarity determining regions of a mouse 21-6 immunoglobulin light chain, and a variable region framework from a human kappa light chain variable region framework sequence except in at least position the amino acid position is occupied by the same amino acid present in the equivalent position of the mouse 21.6 immunoglobulin light chain variable region framework. The humanized heavy chain comprises three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the corresponding complementarity determining regions of a mouse 21-6 immunoglobulin heavy chain, and a variable region framework from a human heavy chain variable region framework sequence except in at least one position the amino acid position is occupied by the same amino acid present in the equivalent position of the mouse 21-6 immunoglobulin heavy chain variable region framework.

## Therapeutic Applications

In this method according to the first aspect of the invention, VLA-4 binding agents, in particular, VCAM fusions and anti-VLA-4 antibody homologs are preferably administered parenterally. The term "parenteral" as used herein includes subcutaneous, intravenous, intramuscular,



intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional and intracranial injection or infusion techniques.

- The VLA-4 binding agents are preferably
- 5 administered as a sterile pharmaceutical composition containing a pharmaceutically acceptable carrier, which may be any of the numerous well known carriers, such as water, saline, phosphate buffered saline, dextrose, glycerol, ethanol, and the like, or combinations thereof.
- 10 The compounds of the present invention may be used in the form of pharmaceutically acceptable salts derived from inorganic or organic acids and bases. Included among such acid salts are the following: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate,
- 15 butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide,
- 20 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, pamoate, pectinate, persulfate, 3-phenyl-propionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate and
- 25 undecanoate. Base salts include ammonium salts, alkali metal salts, such as sodium and potassium salts, alkaline earth metal salts, such as calcium and magnesium salts, salts with organic bases, such as dicyclohexylamine salts, N-methyl-D-glucamine, tris(hydroxymethyl)methylamine and
- 30 salts with amino acids such as arginine, lysine, and so forth. Also, the basic nitrogen-containing groups can be

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quaternized with such agents as lower alkyl halides, such as methyl, ethyl, propyl, and butyl chloride, bromides and iodides; dialkyl sulfates, such as dimethyl, diethyl, dibutyl and diamyl sulfates, long chain halides such as  
5 decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides, aralkyl halides, such as benzyl and phenethyl bromides and others. Water or oil soluble or dispersible products are then obtained.

The pharmaceutical compositions of this  
10 invention comprise any of the compounds of the present invention, or pharmaceutically acceptable derivatives thereof, together with any pharmaceutically acceptable carrier. The term "carrier" as used herein includes acceptable adjuvants and vehicles. Pharmaceutically  
15 acceptable carriers that may be used in the pharmaceutical compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic  
20 acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate,  
25 polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

According to this invention, the pharmaceutical  
30 compositions may be in the form of a sterile injectable preparation, for example a sterile injectable aqueous or

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oleaginous suspension. This suspension may be formulated according to techniques -known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile  
5 injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution.  
10 In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or di-glycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the  
15 preparation of injectables, as do natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant, such as Ph. Helv  
20 or similar alcohol.

The pharmaceutical compositions of this invention, in particular small molecule antagonists of the VLA-4/VCAM-1 interaction, may be given parenterally or orally. If given orally, they can be administered in any  
25 orally acceptable dosage form including, but not limited to, capsules, tablets, aqueous suspensions or solutions. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also  
30 typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn

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starch. When aqueous suspensions are required for oral use, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening, flavoring or coloring agents may also be added.

5 Topically-transdermal patches may also be used. The pharmaceutical compositions of this invention may also be administered by nasal aerosol or inhalation through the use of a nebulizer, a dry powder inhaler or a metered dose inhaler. Such compositions are prepared according to

10 techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other conventional solubilizing or

15 dispersing agents. According to another embodiment compositions containing a compound of this invention may also comprise an additional agent selected from the group consisting of corticosteroids, anti-inflammatories, immunosuppressants, antimetabolites, and immunomodulators.

20 Specific compounds within each of these classes may be selected from any of those listed under the appropriate group headings in "Comprehensive Medicinal Chemistry", Pergamon Press, Oxford, England, pp. 970-986 (1990), the disclosure of which is herein incorporated by reference.

25 Also included within this group are compounds such as theophylline, sulfasalazine and aminosalicylates (anti-inflammatories); cyclosporin, FK-506, and rapamycin (immunosuppressants); cyclophosphamide and methotrexate (antimetabolites); steroids (inhaled, oral or topical) and

30 interferons (immunomodulators).

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The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated, and the particular mode of administration. It should be understood, however, that a specific dosage and treatment regimen for any particular patient will depend upon a variety of factors, including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, rate of excretion, drug combination, and the judgment of the treating physician and the severity of the particular disease being treated. The amount of active ingredient may also depend upon the therapeutic or prophylactic agent, if any, with which the ingredient is co-administered.

The dosage and dose rate of the compounds of this invention effective to prevent, suppress or inhibit cell adhesion will depend on a variety of factors, such as the nature of the inhibitor, the size of the patient, the goal of the treatment, the nature of the pathology to be treated, the specific pharmaceutical composition used, and the judgment of the treating physician. Dosage levels of between about 0.001 and about 100 mg/kg body weight per day, preferably between about 0.1 and about 50 mg/kg body weight per day of the active ingredient compound are useful. Most preferably, the VLA-4 binding agent, if an antibody or antibody derivative, will be administered at a dose ranging between about 0.1 mg/kg body weight/day and about 20 mg/kg body weight/day, preferably ranging between about 0.1 mg/kg body weight/day and about 10 mg/kg body weight/day and at intervals of every 1-14 days. For non-antibody or small molecule binding agents, the dose

range should preferably be between molar equivalent amounts to these amounts of antibody. Preferably, an antibody composition is administered in an amount effective to provide a plasma level of antibody of at least 1 mg/ml. Optimization of dosages can be determined by administration of the binding agents, followed by assessment of the coating of VLA-4-positive cells by the agent over time after administered at a given dose in vivo.

Myeloma cells contained in a sample of the individual's peripheral blood (or bone marrow cells) should be probed for the presence of the agent in vitro (or ex vivo) using a second reagent to detect the administered agent. For example, this may be a fluorochrome labeled antibody specific for the administered agent which is then measured by standard FACS (fluorescence activated cell sorter) analysis. Alternatively, presence of the administered agent may be detected in vitro (or ex vivo) by the inability or decreased ability of the individual's cells to bind the same agent which has been itself labeled (e.g., by a fluorochrome). The preferred dosage should produce detectable coating of the vast majority of VLA-4-positive cells. Preferably, coating is sustained in the case of an antibody homolog for a 1-14 day period.

#### Combination Therapy

In some embodiments of this invention, one or more antagonists of an interaction between an integrin with an  $\alpha 4$  subunit and a ligand for this integrin could be administered in combination with one or more compounds

that are not an antagonist of an interaction between an integrin with an  $\alpha 4$  subunit and a ligand for this integrin.

Multiple myeloma is currently treated  
5 inefficiently with standard chemotherapeutic regimens. In some embodiments of this invention, an antagonist of an interaction between an integrin with an  $\alpha 4$  subunit and a ligand for this integrin could be administered in combination with one or more standard chemotherapeutic  
10 agents. Hence, the two compounds (an antagonist of an interaction between an integrin with an  $\alpha 4$  subunit and a ligand for this integrin and a standard chemotherapeutic agent) could act to prevent or treat multiple myeloma synergistically; or lower dosage of either or both  
15 compounds (an antagonist of an interaction between an integrin with an  $\alpha 4$  subunit and a ligand for this integrin and a standard chemotherapeutic agent) is needed to provide the same effect as a higher dosage of either compound alone.  
20 In some embodiments, the pharmaceutical or prophylactic composition of this invention can also include a pharmaceutically or prophylactically effective amount of a chemotherapeutic agent or another agent, including without limitation: melphalan, bisphosphonates,  
25 thalidomide, erythropoietin, and antagonists, such as mAb blockers, of IL6 and IL15.

#### Animal Models

The animal model has been described in detail (Garrett 1997). Briefly, Radl et al. (1988) had described  
30 a murine model of myeloma which arose spontaneously in

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aged C57BL/KaLwRij mice. This condition occurred in approximately 1 in 200 animals as they aged, and led to a monoclonal gammopathy with some of the features of human disease (Radl 1988). To develop a better and more  
5 reproducible animal model we have established and subcloned a cell line from this murine myeloma called 5TGM1, and found that it causes lesions in mice characteristic of human myeloma, such as severe osteolysis and the involvement of non-bone organs including liver and  
10 kidney (Garrett 1997). Mice inoculated with cultured cells develop disease in a highly predictable and reproducible manner, which includes formation of a monoclonal gammopathy and radiologic bone lesions. Furthermore, some of the mice become hypercalcemic, and  
15 the bone lesions are characterized by increased osteoclast activity. Thus, based on histological examination of affected organs in 5TGM1-bearing mice and increased serum levels of IgG2b, 5TGM1 is defined as a murine myeloma which recapitulates accurately the hallmarks of human  
20 disease.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to be limiting in nature. In the following examples, the necessary restriction enzymes,  
25 plasmids, and other reagents and materials may be obtained from commercial sources and cloning, ligation and other recombinant DNA methodology may be performed by procedures well-known in the art.

#### Example 1: MATERIALS AND METHODS



### 5TGM1 Myeloma Cells

5TGM1 myeloma cells were initially derived from a myeloma which arose spontaneously in aged C57BL/KaLwRij mice (Garrett 1997, Vanderkerken 1997). Cells were grown in Isocove's Modified Dulbecco's Medium (IMDM, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Summit, Fort Collins, CO) and 1% penicillin-streptomycin solution (GIBCO, Grand Island, NY) at 37 C in 5% CO<sub>2</sub> atmosphere. For in vitro experimentation described below, 5TGM1 cells between passage 25 and 30 were used.

### Antibodies, soluble VCAM-1

Neutralizing antibodies against murine VCAM-1 (M/K-2.7), integrin VLA-4 (PS/2 mAb), and Intercellular Adhesion Molecule-1 (ICAM-1, YN1/1.7), were kindly gifted by Dr. Kensuke Miyake (Saga Medical University, Saga, Japan). Recombinant soluble VCAM-1 (Lobb et al., 1991), containing the 7 extracellular domains of human VCAM-1, was the gift of Dr. Roy Lobb, Biogen Inc., Cambridge, MA.

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Using RT-PCR, we confirmed expression of VCAM-1 and integrin  $\alpha$ 4 in bone marrow stromal cells and 5TGM1, respectively. Total RNA was prepared from 5TGM1, a primary culture of bone marrow stromal cells and an ST2 marrow stromal cell line (RIKEN Cell Bank, Tsukuba, Japan) by the single-step RNA isolation method using TRIzol reagent (GIBCO). Three  $\mu$ g of RNA was incubated with 50 ng of random hexamer at 70° C for 10 min and chilled on ice, then converted to first strand cDNA using reverse

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transcriptase (Perkin-Elmer, Branchburg, NJ) according to the manufacturers instruction. The primers used for PCR were as follows: murine VCAM-1 5'-primer; 5'-OH-GCTGCGCGTCACCATTTGTTCTC-3'-OH (SEQ ID NO: 1]; murine VCAM-1  
5 3'-primer, 5'-OH-ACCACCCTCTTGAAGCCTTGTG-3'-OH [SEQ NO: 2]; murine integrin  $\alpha$ 4 5'-primer, 5'-OH-CCCCTCAACACGAACAGATAGG-3'-OH [SEQ ID NO: 3]; murine integrin  $\alpha$ 4 3'-primer; 5'-OH-GCCTTGTCTTAGCAACACTGC-3'-OH [SEQ ID NO:4].

10 PCR was performed for 30 cycles consisting of 1 min at 94° C, 1 min at 55° C and 2 min at 72° C. PCR reaction mixture (total 50  $\mu$ l) contained 10 microliters. First strand cDNA, 50 mM KCL, 10 mM Tris-HCL (pH 8.3), 2 mM MgCl<sub>2</sub>, deoxy-NTP mix (0.2 mM each), the pair of primers  
15 (0.15 micromolar each) and 2 U Taq DNA polymerase (Perkin-Elmer, Branchburg, NJ). The PCR products were separated on 2.5% agarose gels containing ethidium bromide and visualized under ultraviolet light. The size of the fragments were confirmed by reference to molecular weight  
20 markers.

#### **Attachment of 5TGM1 Cells onto Bone Marrow Stromal Cells**

For heterotypic cell-cell adhesion assays, ST2 cells (5 e 4/well) were seeded in 48-well culture plates (Costar, Cambridge, MA) and cultured 48 h in alphaMEM  
25 supplemented with 10% FBS until confluency. 5TGM1 cells (5 e 6) were labeled by incubation with 10 microCi [methyl-3H] thymidine (New England Nuclear) for 24 h at 37° C in the culture medium. After the ST2 monolayer was formed, it was incubated with 1% bovine serum albumin  
30 (BSA, Sigma, St Louis, MO) in serum-free alphaMEM for 1

hour and tritium-labeled 5TGM1 cells were plated onto the monolayer. The system was incubated in the absence or presence of antibodies to VCAM-1 or  $\alpha 4\beta 1$  integrin at 37° C for 1 h. Non-adherent cells were removed by washing  
5 with 5% trichloroacetic acid twice and PBS twice, and adherent cells were solubilized in 300 microliters of 0.25 mM NaOH, neutralized with the same volume of 0.25 mM HCl and the radioactivity was determined in a liquid scintillation counter.

10 **Osteoclast Formation Assay in the Co-culture of 5TGM1 and Mouse Bone Marrow Cells**

Mouse bone marrow cells were obtained from 5-week-old male C57BL mice as described previously (Yoneda 1993). Femurs and tibiae were dissected aseptically and  
15 both ends cut off. Bone marrow cells were flushed out, collected and incubated in alphaMEM supplemented with 10% FBS (Hyclone, Logan, UT) and 1% penicillin-streptomycin in 100 mm-culture dishes (Becton Dickinson Labware, Bedford, MA) at 37°C for 2 h. Non-adherent cells containing  
20 hemopoietic osteoclast precursors and stromal cells were harvested. Bone marrow cells ( $1 \times 10^6$ ) and 5TGM1 cells ( $1 \times 10^3$ ) in 300 microliters of the culture medium were plated onto 48-well culture plates (day 0). On day 2, 300 microliters of fresh culture medium was gently added to  
25 each well, and on day 4, 300 microliters of spent medium was replaced with the same volume of fresh medium. On day 6, the cultures were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using commercial kits (Sigma). TRAP-positive multinucleated  
30 cells with more than 3 nuclei were defined as

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osteoclast-like (OC-like) cells, and manually counted under microscope. To confirm that these OC-like cells have the capability to resorb bone, 5TGM1 cells and marrow cells were co-cultured on 5x5 mm whale dentine slices in the same condition, and resorption pits formed on these dentine slices were examined by scanning electron microscopy as described (Yoneda 1992).

In some experiments, co-cultures of 5TGM1 myeloma cells and marrow cells were performed using transwell inserts (Becton Dickinson Uware) to prevent direct contact between these two types of cells. (2 e 6, 24-well plates, Costar). Marrow cells were plated in the lower chambers and 5TGM1 myeloma cells (2 e 3) were then plated in either lower (direct contact) or upper (no contact) chambers.

#### **Organ Cultures of $^{45}\text{Ca}$ -labeled Fetal Rat Long Bones**

Conditioned media harvested from 5TGM1 cultures were assayed for bone-resorbing activity by organ cultures of  $^{45}\text{Ca}$ -labeled fetal rat long bones as described previously (Mbalaviele 1995). Pregnant rats were injected with 250  $\mu\text{Ci}$  of  $^{45}\text{Ca}$  (New England Nuclear) on the 18th day of gestation. Radius and ulna bone shafts were obtained from 19-day fetuses by microdissection, and precultured for 24 h in BGJ medium (Sigma) supplemented with 0.1% BSA between air and liquid-phase on stainless mesh grids. Bones were then cultured in the presence of conditioned media (50% v/v) or in control medium for 120 hours. The media were changed once at 48 hours. At the end of the culture, bones were incubated in ice-cold 5% trichloroacetic acid for 2h, and  $^{45}\text{Ca}$  radioactivity in

bones and media determined in a liquid scintillation counter. Bone resorption was quantitated as the percentage of  $^{45}\text{Ca}$  released into the medium from bones as calculated by:  $(^{45}\text{Ca count in medium}) / (^{45}\text{Ca count in medium and bone}) \times 100$ .

#### **Co-culture of 5TGM1 Myeloma Cells with Mouse Stromal Cell Line ST2 Cells**

ST2 cells ( $0.5 \times 10^6$ ) and 5TGM1 ( $4 \times 10^6$ ) cells were plated together onto 60-mm culture dishes (Becton Dickinson) in 10% FBS-supplemented IMDM and cultured overnight, washed with serum-free IMDM twice, and incubated in 5 ml of serum-free IMDM. After 48 h, conditioned media were harvested and stored at  $-70^\circ \text{C}$  until use.

#### **Effect of mAb PS2 to VLA-4 on serum IgG2b elevation in 5TGM1-bearing mice**

Mice were injected with  $1 \times 10^5$  5TGM1 cells, which were allowed to colonize the bone marrow. Mice were split into two groups of three, one serving as a control group, and the second treated biweekly beginning on day 8 with 80  $\mu\text{g}$  mAb PS/2 (4 mg/kg). Levels of IgG2b, the antibody isotype produced by 5TGM1 myeloma cells, were measured weekly from weeks 1 to 6.

### **RESULTS**

#### **Expression of VCAM-1, VLA-4, and effect of Antibodies Against VCAM-1 and VLA-4 on 5TGM1 Attachment to ST2 Monolayers**

Using RT-PCR, we confirmed the expression of VCAM-1 and integrin VLA-4 in bone marrow stromal cells and myeloma cells, respectively. As expected, both the ST2 stromal cell line and primary bone marrow stromal cells expressed VCAM-1, while 5TGM1 did not. In contrast, the 5TGM1 myeloma cells expressed integrin VLA-4, whereas stromal cells did not (data not shown). In addition, both anti-VCAM-1 antibody (10 µg/ml) and VLA-4 antibody (10 µg/ml) partially (50-80%) inhibited the attachment of 5TGM1 cells to ST2 monolayers, showing that VCAM-1 and the VLA-4 integrin expressed on these cells are biologically functional and that these antibodies have neutralizing activity (data not shown).

#### **OC-like Cell Formation in the Coculture of 5TGM1 Myeloma Cells with Mouse Bone Marrow Cells**

On day 6 of the coculture of 5TGM1 cells and mouse marrow cells, numerous TRAP-positive multinucleated osteoclast-like (OC-like) cells were formed. These OC-like cells exhibited resorption pit formation on dentine slices, demonstrating that these cells were capable of resorbing bone, and possess an osteoclastic phenotype. In experiments using transwell inserts, formation of OC-like cells was observed when 5TGM1 cells were cultured in direct contact with bone marrow cells. In contrast, there was only a marginal number of OC-like cells formed when 5TGM1 cells were separated from marrow cells by the transwell membrane. Thus 5TGM1 cells induce osteoclast formation in mixed marrow cultures, and this induction requires direct cell-cell contact.

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### Effect of Antibodies Against VCAM-1 and Integrin VLA4 on OC-like Cell Formation in the Co-culture of 5TGM1 and Marrow Cells

Both anti-VCAM-1 antibody (VCAM-1 Ab, 10 µg/ml) and anti VLA-4-integrin antibody ( $\alpha 4\beta 1$  Ab, 10 µg/ml) dramatically inhibited OC-like cell formation. In contrast, mAb against ICAM-1, another adhesion molecule on marrow stromal cells implicated in stromal/myeloma interactions, had no effect on OC-like cell formation (Figure 1).

To determine whether this inhibition by VCAM-1 and VLA-4 mAbs was specific for 5TGM1-induced OC-like cell formation and was not due to cytotoxicity, the effects of these antibodies were examined on OC-like cell formation induced by  $1,25 (\text{OH})_2\text{D}_3$ , a widely-used stimulator of osteoclastogenesis in mouse bone marrow cell cultures (Takahashi 1988). Neither VCAM-1 Ab nor VLA-4 mAb inhibited OC-like cell formation induced by vitamin D3, which itself had no effect on VCAM-1 expression in stromal cells (data not shown).

### Effect of conditioned Medium Harvested from the Co-culture of 5TGM1 and ST2 on Bone Resorption

Conditioned medium from the co-culture of 5TGM1 cells and ST2 cells showed a marked increase in bone resorption in the fetal rat long bone assay (Figure 2), while conditioned medium of 5TGM1 caused only a marginal increase, as compared to control medium. Conditioned medium from ST2 cells showed no increase in bone resorption. Thus direct cell-cell contact via VCAM-1 and

VLA-4 both induces osteoclast-like cells and production of bone-resorbing factors in vitro.

**Effect of Recombinant Soluble VCAM-1(sVCAM-1) on the  
Production of Bone-resorbing and Osteoclastogenic Activity  
5 by 5TGM1 Cells**

Conditioned medium of 5TGM1 treated with a soluble recombinant form of VCAM-1(sVCAM-1) increased bone resorption in fetal rat long bones in a dose-dependent manner, while conditioned medium obtained from untreated  
10 5TGM1 only marginally increased bone resorption. Soluble VCAM-1 itself had no effects on bone resorption (data not shown). In the mouse marrow culture system, conditioned medium harvested from 5TGM1 cells treated with sVCAM-1 showed increased activity of OC-like cell formation, while  
15 conditioned medium of untreated 5TGM1 exhibited only marginal activity of OC-like cell formation (Figure 3).

**Expression of Rank ligand mRNA in marrow stromal cells  
(ST2) cultured in the presence and absence of murine  
myeloma cells**

20 Because Rank ligand appears to be an important mediator of OCL formation and may be the final common pathway for the effects of osteoclastogenic cytokines on OCL formation, we have examined the expression of Rank ligand in 5TGM1 and ST2 cells both individually and when  
25 cocultured. We find that coculture of 5TGM1 and ST2 cells induces Rank ligand mRNA in the ST2 cells. Furthermore, while 5TGM1 cells do not express Rank ligand, they do so when treated with sVCAM-1 (not shown). Finally, the conditioned medium from 5TGM1 cells treated with sVCAM-1

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induced Rank ligand mRNA in ST2 cells, suggesting that the VCAM-1/VLA-4 pathway produces a cytokine in myeloma cells that enhances Rank ligand expression by marrow stromal cells (data not shown).

5           In summary, we show that 5TGM1 cells alone  
produce marginal amount of activity that stimulates  
OC-like cell formation and bone resorption. However, when  
5TGM1 myeloma cells were co-cultured with bone marrow  
cells containing hemopoietic osteoclast precursors and  
10 stromal cells, they strongly adhered to the stromal cells  
and increased OC-like cell formation. There were no  
OC-like cells formed in the co-cultures in which 5TGM1  
cells were prevented from contacting stromal cells.  
Furthermore, in organ cultures of fetal rat long bones the  
15 conditioned medium harvested from the cocultures of 5TGM1  
myeloma cells and ST2 bone marrow stromal cells had  
increased bone resorbing activity compared with  
conditioned medium of either ST2 or 5TGM1 alone. These  
data are consistent with the notion that direct cell-cell  
20 contact of 5TGM1 cells with bone marrow stromal cells is  
required for the production of osteoclast-stimulating and  
bone-resorbing activity. We then determined what cell  
adhesion molecules were involved in the direct cell-cell  
interaction between 5TGM1 cells and marrow stromal cells  
25 that is necessary for the production of osteoclastogenic  
activity. Our data indicate that VCAM-1 and VLA-4  
integrin play a role in this cell-cell interaction, since  
neutralizing antibodies to these two adhesion molecules  
profoundly decreased OC-like cell formation in the  
30 co-cultures. The VCAM-1/VLA-4 integrin interaction is

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responsible for the cell-cell communication between marrow stromal cells and 5TGM1 myeloma cells leading to increased production of a osteoclastogenic and bone-resorbing activity. Finally, this bone resorbing activity in part  
5 is due to induction of Rank ligand.

### Example 2: IN VIVO EXPERIMENTS

Our in vitro studies suggest that the interaction between VLA-4 on myeloma cells with VCAM-1 on marrow stromal cells may play a key role in the induction  
10 of bone resorbing activity by myeloma. We have taken the key step of testing this hypothesis in vivo in an animal model which accurately reflects human disease.

A. In this experiment, mice were injected with 1 e 5 5TGM1 myeloma cells, which were allowed to colonize  
15 the bone marrow. Mice were split into two groups of three, one serving as a control group, and the second treated biweekly beginning on day 8 with mAb PS/2. Levels of IgG2b, the antibody isotype produced by 5TGM1 myeloma cells, were measured weekly from weeks 1 to 6. Treatment  
20 with mAb at a dose of 80 µg per injection (~4 mg/kg) biweekly strongly inhibited IgG2b production, indicative of significant inhibition of myeloma cell survival and growth in vivo (Figure 4). Further, the treated mice showed reduced incidence of paraplegia (all 3 untreated  
25 animals showed paraplegia on day 42, while only one of the treated animals showed paraplegia). The two treated animals with no paraplegia also showed a reduction in spleen and liver weights, which also correlate with tumor burden. Finally, the treated animals showed a reduction

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in tumor area by histology (from 6.71 +/- 1.74 to 0.05 +/- 0.08 square millimeters) in the tibia and femurs. There was no effect of treatment on serum calcium levels (data not shown)

5                   B.    In a parallel experiment, treatment with 40 µg PS/2 mAb biweekly had no effect on IgG2b levels (not shown). These data show that mAb PS/2 to VLA-4 strongly inhibits the growth of established myeloma cells in a dose-dependent fashion.

10                   C.    In another in vivo experiment, 18 SCID mice were injected with 5TGM1 myeloma cells at day 0. Four mice were treated with PBS; 4 mice were treated in a prophylactic protocol with mAb M/K 2.7 reactive against to mouse VCAM1 at a dosage of 80 µg (-4 mg/kg) every 3 days  
15 starting at day -1 (i.e. days - 1, 2, 5, 8, and 11). In a parallel experiment using the same protocol, five mice were treated with 160 µg mAb M/K 2.7. In addition, five mice were treated with 160 µg mAb M/K 2.7 starting at day 8 (i.e. days 8, 11, 14, 17, and 20) in a therapeutic  
20 protocol. Serum was taken from all mice on days 21, 28, and 35, and animals were X-rayed then sacrificed for histology on day 35. All three treatment groups showed a reduction in serum IgG2b levels, indicative of reduced myeloma cell burden (Figure 5). A significant effect was  
25 also observed on spleen weights at the low dose prophylactic protocol relative to control (0.23 +/- 0.14 g for control versus 0.08 +/- 0.04 for treated). In the prophylactic high dose group 4 of 5 animals showed a clear reduction in spleen weight, but the overall value was not

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significant because of one animal with a large spleen weight (data not presented).

D. One can investigate whether an initial high bolus dose of  $\alpha 4$  integrin antagonist, followed by a maintenance dose, improves efficacy. The myeloma cells are already established in the marrow compartment, and their tight VLA-4-dependent interaction with VCAM-1 needs to be inhibited. Furthermore, presumably the greater the number of established myeloma cells, the higher the initial dose required to flush cells out into the peripheral circulation.

A larger study with the anti-VLA-4 antibody PS/2 was therefore performed. Twenty eight SCID mice were injected with 5TGM1 myeloma cells at day 0. Nine mice received no treatment; 9 mice received an isotype-matched control IgG mAb; 10 mice were treated with mAb PS/2 to alpha 4 integrin. A different therapeutic regimen was given, in which mice were given a high dose of mAb (200  $\mu$ g) on days 4, 5, and 6, then a maintenance dose of 80  $\mu$ g (-4 mg/kg) every 3 days starting at day 8.

There was a statistically significant reduction in serum IgG2b when the treated group was compared to either the untreated or control IgG-treated group at weeks 3 and 4 (data not presented). Importantly, when the treated group was compared to either the untreated or control IgG-treated group there was a clear effect on survival (Figure 6).

### Example 3: OTHER IN VIVO EXPERIMENTS

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Based on the information presented herein for the first time, persons having ordinary skill in the art can readily confirm and extend the importance of the  $\alpha 4$  integrins and their ligands in multiple myeloma using the murine animal model described.

The following series of experiments are well within the level of skill in the art based upon the present disclosure but serve merely to exemplify, and not limit, the types of work.

- 1) Dose response to mAb PS/2 to determine the optimal biweekly maintenance dose. 80  $\mu$ g shows good efficacy, but 40  $\mu$ g was without effect. One examines higher doses up to 20 mg/kg two or three times weekly to determine optimal dosing.
- 2) Patients present with disease at different stages of severity, linked to increased tumor burden. One examines the efficacy of mAb PS/2 given at different times after establishment of disease, i.e., one compares treatment initiation at 8 days (see for example Figure 4) to initiation after two, three, four and five weeks post inoculation to see how late mAb can be given to provide sonic relief of symptoms.
- 3) The effects of mAb MK-2 to murine VCAM-1 are examined, following the same parameters outlined above (dosing, timing of dosing) for mAb to VLA-4. It is anticipated that similar dosing levels will be required to see efficacy.
- 4) Further markers of myeloma progression are examined, including tumor burden in both marrow and extramedullary sites, quantification of bone lesions

by radiographic analysis of the skeleton by histomorphometry; measurement of rates of bone repletion by evaluation of collagen crosslinks in plasma; measurement of monoclonal protein production in plasma; hypercalcemia where present; and mortality.

- 5
- 5) Multiple myeloma is currently treated inefficiently with standard chemotherapeutic regimens. The additive or synergistic effects of mAbs at optimal dosing in conjunction with, or either before or after, dosing with appropriate chemotherapeutic regimens is examined.
- 10
- 6) The ability of a small molecule  $\alpha 4$  integrin inhibitor that is selective for one particular  $\alpha 4$  integrin or is selective for several  $\alpha 4$  integrins at once or the ability of combinations of such inhibitors, to mimic the effects of mAbs and block myeloma progression is examined using the protocols and outcomes described above. Small molecule
- 15
- 20 inhibitors are delivered parenterally or orally, in the dosing range of 0.1 to 30 mg/kg, once or twice daily, or twice or three times weekly.

**Example 4: ANTI- $\alpha 4$  INTEGRIN ANTIBODY ENHANCED SENSITIVITY OF MYELOMA CELLS TO MELPHALAN (CHEMOTHERAPY COMBINATION EXPERIMENTS)**

25

Cell-cell contact of myeloma cells with stromal cells via  $\alpha 4\beta 1$  integrin and VCAM-1 apparently facilitate their arrest, proliferation, survival and production of osteoclast activating factors in bone marrow cavity.

30 Therefore, interference with stromal cell/myeloma cell

interactions is a potential adjuvant intervention to enhance the efficacy of anti-cancer agents in myeloma bone disease.

Here, we studied the effects of a neutralizing  
5 antibody to  $\alpha 4$  integrin ( $\alpha 4$ Ab), which disrupts stromal  
cells/myeloma cell interactions, on the sensitivity of  
myeloma to melphalan, a most widely-used chemotherapeutic  
agent for myeloma, using the 5TGM1 mouse myeloma cells  
that reproducibly cause extensive osteolysis in tumor-  
10 bearing animals. The  $\alpha 4$ Ab (100-200  $\mu$ g/mouse, ip, 2 or 3  
times a week) and melphalan (50, 100, 200  $\mu$ g/mouse, ip,  
once a week) were administered following inoculation of  
5TGM1 cells in the tail vein in male xid-nu-bg mice.  
Melphalan alone at doses of 50 and 100  $\mu$ g/mouse failed to  
15 suppress serum IgG2 levels, a systemic indicator of  
myeloma tumor burden, whereas serum IgG2 levels were  
significantly suppressed by 200  $\mu$ g/mouse melphalan.  
Combined treatment with melphalan (50 or 100  $\mu$ g/mouse)  
and  $\alpha 4$ Ab suppressed serum IgG2 levels to a greater extent  
20 than melphalan (200  $\mu$ g/mouse) alone. Moreover,  
histomorphometric examination revealed that melphalan (50  
 $\mu$ g/mouse) combined with  $\alpha 4$ Ab significantly decreased  
5TGM1 tumor volume in bone compared with melphalan (200  
 $\mu$ g/mouse) alone.  
25. To study the role of cell-cell contact of 5TGM1  
cells with marrow stromal cells in the sensitivity to  
melphalan, the effects of melphalan on 5TGM1 cells  
cultured in contact with the ST2 mouse marrow stromal  
cells were examined. We found that 5TGM1 cells cultured  
30 on ST2 cells exhibited increased survival and reduced  
apoptosis in the presence of melphalan compared with 5TGM1

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cells cultured on tissue culture plates. Furthermore,  $\alpha 4$ Ab reduced survival and increased apoptosis in 5TGM1 cells in these cultures. In summary, our data show that  $\alpha 4$ Ab enhanced the sensitivity of 5TGM1 cells to melphalan and suggested that disruption of stromal cell/myeloma cell interactions using  $\alpha 4$ Ab is an effective adjuvant therapy, allowing dosage reduction of chemotherapeutic agents and thereby lowering the risk of adverse effects.

In one particular set of experiments on the combined effects of melphalan and anti-alpha 4 integrin Ab in 5TGM1 model, as shown in an experimental protocol in Figure 7, an initial high dose of 200  $\mu$ g mAb was given during the first week, followed by a maintenance dose of 100  $\mu$ g. Melphalan was given three times. Animals were sacrificed at day 28 and serum IgG2b levels (a surrogate marker of tumor burden) and tumor burden in bone were measured. The serum IgG2b was only modestly affected by either melphalan or anti- $\alpha 4$  mAb, while the combination produced a significant drop in IgG2b levels (Fig. 8). The tumor volume in bone marrow, measured as a ratio of treated to untreated (so that a reduction results in <100%), was also reduced (Fig. 9). The data were a summary of two experiments combined.

We also examined the effects of the combined administration of melphalan (100  $\mu$ g/mouse) and PS/2 mAb on survival of 5TGM1-bearing mice. An experimental protocol of these experiments is shown in Figure 35. We evaluated survival by the onset of hindleg paralysis (i.e., the onset of hindleg paralysis was the end-point in these experiments). As shown in Figure 36, melphalan (100  $\mu$ g/mouse) alone had no effect on the onset of hindleg

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paralysis and  $\alpha$ 4Ab slightly but significantly delayed the onset of hindleg paralysis. Combined administration of melphalan and Ab significantly delayed the onset of hind-leg paralysis. Kaplan-Meier analysis of these data  
5 demonstrated that the effects of  $\alpha$ 4Ab and the combination of  $\alpha$ 4Ab and melphalan were significantly different from the untreated. Melphalan alone, at a dose of 200  $\mu$ g/mouse, significantly delayed the onset. Thus, we can reduce the dose of Melphalan to a quarter in the presence  
10 of the Ab, as compared to melphalan alone. These data suggested that combined use of the  $\alpha$ 4Ab enables the reduction of the dose of melphalan, thereby decreasing adverse effects.

We also wish to study the dose-response of  
15 melphalan with a fixed dose of an anti- $\alpha$ 4 mAb.

**Example 5: IN VIVO EXPERIMENTS CONDUCTED IN  
IMMUNO-COMPETENT RAJ MICE**

Results reported below indicated a beneficial therapeutic effect of treatment with the PS/2 mAb in a  
20 mouse model of myeloma when initiated on day four after infusion of myeloma cells. Mice were treated with PS/2 mAb periodically and were sacrificed for analysis on day 20 and 21.

These experiments showed an effect of the PS/2  
25 mAb on a number of disease parameters treatment relative to untreated controls:

1. Splenomegaly was reduced by 24% and tumor burden in the spleen was reduced by 75%. A disease dependent

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increase in non-myeloma cells was not affected by PS/2 mAb treatment.

2. Tumor burden in the bone marrow was reduced by 22%.
3. Myeloma cells in the peripheral blood was reduced by 57%.
4. Increased LDH levels were reduced by 34% and AST levels were reduced by 42%. Phosphorus levels, which decrease slightly with disease, improved by 13%.
5. Circulating mIgG2b levels were reduced by 50% .

At the conclusion of the experiment, circulating levels of PS/2 mAb were undetectable in most mice. This indicated a possible reduction in the effectiveness of mAb treatment, probably either due to an immune response to the rat IgG or the antibody were bound up by the expanding myeloma population.

#### Methods

C57BL/KaLwRaj mice were inoculated, via the tail vein, with  $1 \times 10^6$  5TGM1 myeloma cells. The mice were randomly divided into three groups of ten animals each.

One group was left untreated, one group received PS/2 mAb treatment and the third group received rat IgG2b isotype control mAb. The antibody treated groups received 200 micrograms of antibody on days 4,5,and 6. After that the mice were injected twice weekly with 200 micrograms of antibody. The mice were sacrificed for analysis on days 20 and 21. Blood was removed via cardiac puncture for FACS analysis of cell populations. The plasma was used for quantification of circulating mIgG2b, PS/2 mAb and numerous blood chemistry parameters. The spleen was

25            Circulating PS/2 mAb levels were determined by a FACS binding assay. 5TGM1 cells, grown in vitro, were incubated with plasma from treated animals. The bound antibody was then detected with labeled anti rat Fc antibody.

## 30 Results

5)

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25

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expanding extramedullary hematopoietic cell population due to the physiological stress of bone marrow neoplasia.

Figure 12 shows representative FACS plots of the staining for lineage markers and cytoplasmic mIgG2b in splenocytes. The reduction in mIgG2b positive cells was clearly shown.

By using the tumor burden calculation to assess the effect of PS/2 mAb treatment in the spleen, the reduction was 75%. The effect of PS/2 mAb treatment was significant ( $p < .001$ ) when compared to either control group. These results are shown in Figure 13.

Figure 14 shows that, at this point in disease development, 77% of bone marrow cells were now mIgG2b positive myeloma cells. PS/2 mAb treatment resulted in a modest 15% reduction in the percentage of cytoplasmic mIgG2b positive cells in the bone marrow while there was a 6% reduction in the rat IgG2b isotype mAb control group. Analysis by Student t test showed that the reduction of mIgG2b positive cells in the PS/2 mAb treatment group was significant ( $p < .001$ ) when compared to untreated group or the rat IgG2b isotype mAb control group ( $p < .05$ ). As in the spleen, the mIgG2b positive population was split between a lineage positive and a lineage negative population and the effect of PS/2 mAb was on both populations. Figure 14 also shows a disease dependent depletion in the percentage of non-myeloma cells (defined as mIgG2b negative) in the bone marrow of all treatment groups. A higher percentage of bone marrow cells were retained in PS/2 mAb groups as compared to the untreated or rat IgG2b isotype mAb control groups. The PS/2 mAb treatment was significant when compared to the untreated

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control group ( $p < .001$ ) and the rat IgG2b control group ( $p < .05$ ).

Figure 15 shows the results of the tumor burden calculations for the bone marrow. These results showed that there was a 22% reduction in the absolute number of mIgG2b positive cells and no effect of the rat IgG2b mAb isotype control. The effect of PS/2 mAb was significant ( $p < .05$ ) when compared to either the untreated or rat IgG2b control groups.

The Experimental Pathology Report of the H+E stained sections indicated that there were distinctly fewer neoplastic infiltrates within the liver and spleen of PS/2 mAb treated animals as compared to either the untreated or rat IgG2b control groups. In the bone marrow, no distinctly apparent differences were noted among the groups.

Figure 16 shows that mIgG2b positive myeloma cells were detected in the peripheral blood and PS/2 mAb treatment reduces their numbers. The 57% reduction in mIgG2b positive cells with PS/2 mAb treatment was significant ( $p < .001$ ) when compared to either the untreated or the rat IgG2b control groups.

Figure 17 shows the results of the blood chemistry study. As previously observed, there was an increase in LDH and AST levels associated with myeloma. PS/2 mAb treatment significantly reduced the levels of both enzymes as shown by a Student t test p value  $< .001$ . There was no effect of the isotype control rat IgG2b antibody on these enzymes. Mice with myeloma also had a slight decrease in phosphorus levels. PS/2 mAb treatment but not isotype control rat IgG2b mAb reduced this

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decrease. The PS/2 mAb effect on the phosphorus levels was significant with a Student t test p value of  $<.05$

Circulating mIgG2b levels were measured by ELISA. Figure 18 shows that at the conclusion of the experiment the untreated group had an average of 2.4 mg/ml of circulating antibody while the PS/2 mAb group had a 50% reduction to 1.2 mg/ml. There was a small reduction in the control rat IgG2b group to 2.0 mg/ml. The reduction in the PS/2 mAb group was significant with a Student t test p value of  $<.05$  as compared to either the untreated or rat IgG2b control groups. The reduction in the rIgG2b control group was not significant.

Results of the FACS binding assay, to determine circulating PS/2 mAb levels, showed that only two of the ten animals that received PS/2 mAb treatment had detectable levels of PS/2 mAb in the plasma. This result indicated that the amount of circulating PS/2 mAb was limited. This suggested that the PS/2 mAb was either fully bound to the myeloma cells or that the antibody was cleared by an immune response.

In summary, administration of PS/2 mAb in this mouse model system of multiple myeloma had the following effects: splenomegaly was reduced 24%, tumor burden in spleen by 75%, tumor burden in marrow by 22% (Fig. 6), circulating myeloma cells by 57% (Fig. 7, left hand panel [IgG2b (pos) cells are the myeloma cells]), reduced liver enzyme levels were normalized, suggesting a reduction in liver tumor burden, and circulating plasma levels of IgG2b were reduced 50%.

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To evaluate the effects of mAb treatment at later times, three 'acute' treatment protocols were tried (Fig. 19). Mice were injected with 5TGM1 cells, and then given mAb PS/2 on day 16 (1 day treatment), days 16 and 17 (2 day treatment), or days 14-19 (6 day treatment).

Remarkably, as shown in Figure 20, the percentage of IgG2b-positive (i.e. myeloma) cells was dramatically reduced in spleen and blood with either 1 or 2 day treatments. The tumor burden (i.e. the absolute number of myeloma cells, rather than the % of total cells) was also reduced by both treatments (Fig. 21). The 1 or 2 day treatments had no effect on cells in bone marrow, however, whether expressed as % (Fig. 20) or tumor burden (not shown). The six day protocol reduced cells in both blood and spleen, as expected (Fig. 22). The percentage of cells in bone marrow is unaffected, but there was a clear effect when expressed as tumor burden (Fig. 23) because the total cell number and myeloma cell numbers were reduced in proportion.

These experiments are key because they suggest that: 1) very late therapeutic intervention can be successful; 2) the therapeutic effect of the mAb in the longer term experiments may be lost over time, due to the intact immune response to the rat antibody in the Raj mice; 3) the rapid drop in myeloma cell numbers in blood and spleen is likely not due to decreased growth but through effects on other parameters, such as apoptosis.

#### **Chemotherapy combination experiments**

Additive/synergistic effects of the combination of PS/2 mAb and melphalan were seen under most conditions.



The data were only useful on day 27. Figure 24 shows a general experimental protocol. Figure 25 shows that IgG2b levels were unaffected by PS/2 mAb or melphalan alone, but reduced ( $p < 0.05$ ) by the combination. Figure 26 shows that the percentages of GFP+ myeloma cells in blood and spleen were unaffected by melphalan but reduced by PS/2 mAb. The combination further reduced levels in spleen, but was not statistically significant in blood because PS/2 mAb alone was so effective. Neither melphalan nor PS/2 mAb alone affected percentage of GFP+ myeloma cells in bone marrow, but the combination was effective ( $p < 0.05$ ). If expressed as tumor burden, the combination was once again statistically significantly better than monotherapy in both spleen and bone marrow (Figure 27). Figure 28 shows in tabular forms that PS/2 mAb plasma levels are undetectable in all but one assay.

**Example 6: IN VIVO EXPERIMENTS CONDUCTED IN  
IMMUNO-COMPETENT RAJ MICE: VLA4 SMALL  
MOLECULE EXPERIMENTS**

To evaluate the efficacy of VLA4 binding small molecules, BIO8809, and a related but non-VLA4 binding chemical control, BIO9257, were used. These are PEGylated small molecules with extended half lives. BIO8809 was described in application WO 01/12186, the disclosure of which is hereby incorporated by reference. The chemical syntheses and the structures of BIO8809 and BIO9257 are shown in Example 7.

In two experiments, BIO8809 showed statistically significant inhibition of myeloma cell numbers in both blood and spleen, but not in bone marrow.

In a long-term treatment experiment (the protocol is summarized in Figure 29), PS/2 mAb had the expected effect on the percentage of myeloma cells in blood and spleen, and surprisingly, in this experiment, on bone marrow as well (Fig. 30). The small molecule had statistically significant effects in spleen (at all doses), and in blood (0.03 mg/kg dose only), but not in bone marrow at any dose. Replotting as tumor burden did not affect the data (Fig. 31).

In an acute 6 day treatment experiment (see protocol summarized in Figure 32; 3 day treatment for small molecules), PS/2 mAb had the expected effect on the percentage of myeloma cells in blood and spleen (Fig. 33). In this experiment, the untreated control in bone marrow was very low, making these data uninterpretable (Fig. 33). The small molecule had statistically significant effects in spleen (at all doses, with nice dose response), and in blood (all doses versus BIO9257 control; 3 mg/kg dose only versus untreated control). In bone marrow no dose showed significant inhibition, even versus BIO9257 control. Replotting as tumor burden did not affect the data (Fig. 34).

The small numbers (N=2 per dose) in these preliminary experiments precluded more robust analysis or interpretation. Nevertheless, these data already show that the small molecule inhibitors can work as effectively as the mAb in reducing blood and spleen myeloma burden.

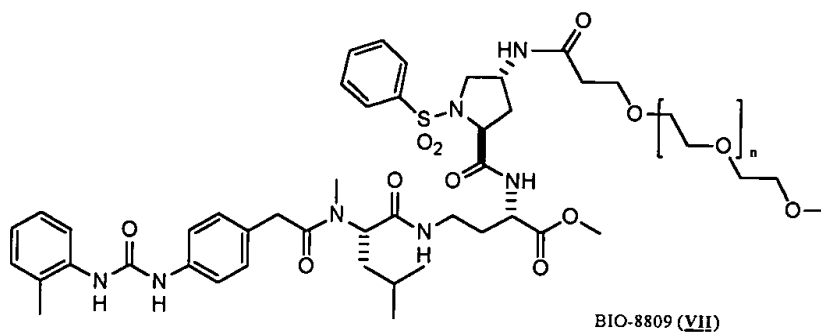
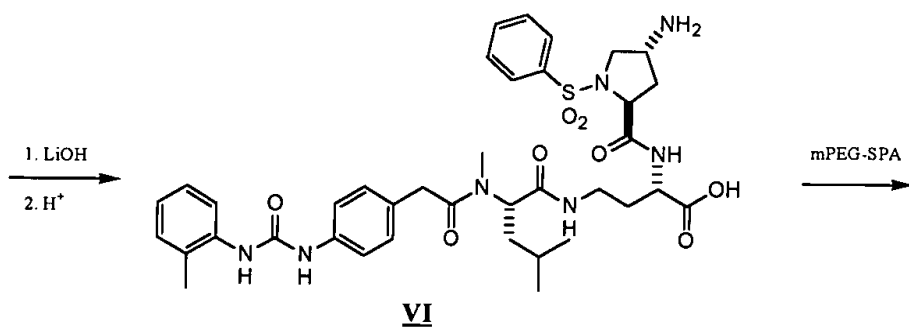
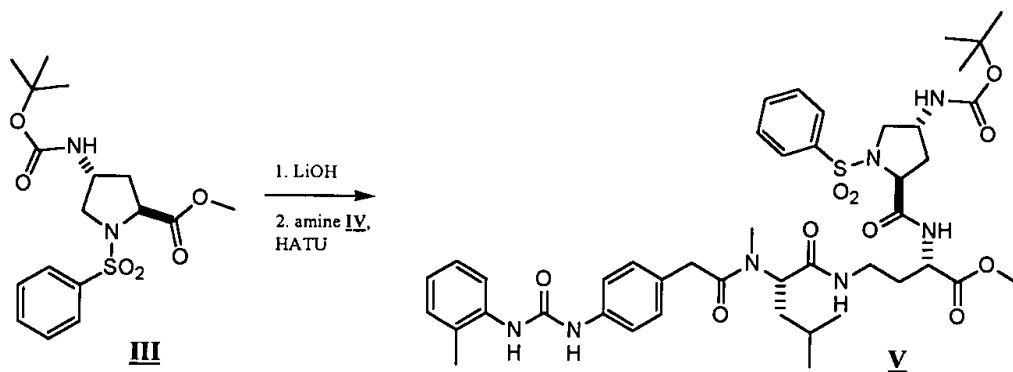
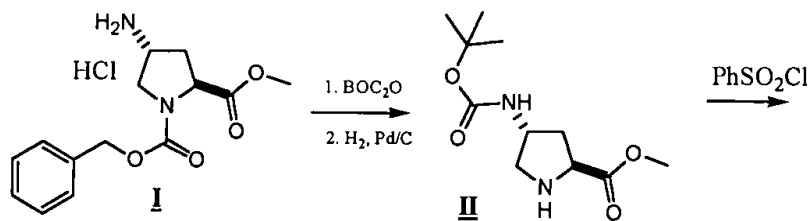
**Example 7: BIO-8809 AND BIO-9257**

(2S,4R)-4-amino-1-[benzyloxycarbonyl]pyrrolidine  
-2-methylcarboxylate hydrochloride (I) could be converted

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to the 4-*tert*-butoxycarbonyl derivative II by protection  
of the 4-amino group with di-*tert*-butyldicarbonate and  
subsequent removal of the 1-benzyloxycarbonyl group using  
hydrogenation over Pd/C catalyst. Reaction with  
5 benzenesulfonyl chloride gave sulfonamide III. Hydrolysis  
of the methyl ester with lithium hydroxide gave the acid,  
which was coupled to amine salt IV using  
[O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium  
hexafluorophosphate] (HATU) to give V. This methyl ester  
10 was hydrolyzed with lithium hydroxide, followed by the  
deprotection of the *tert*-butoxycarbonyl protected 4-amino  
group using acidic conditions to give VI. This amino acid  
was then coupled to methoxy-poly(ethylene  
glycol)-succinimidyl propionate (MW: 20,000) to give  
15 BIO-8809 (VII).

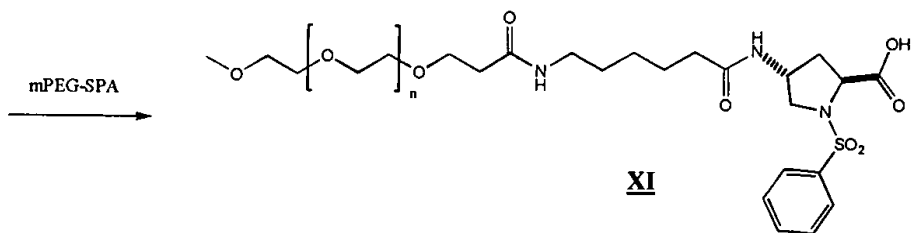
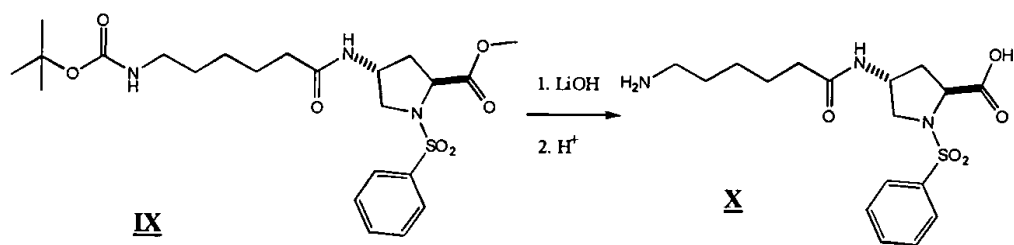
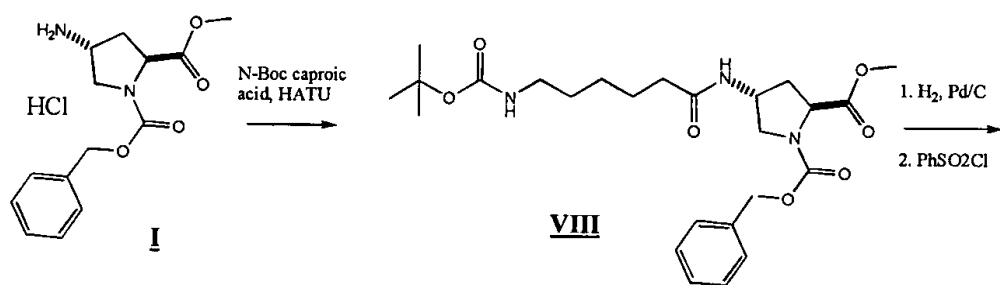
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BIO-8809 (**VII**)

(2S,4R)-4-amino-1-[benzyloxycarbonyl]pyrrolidine  
-2-methylcarboxylate hydrochloride (I) was coupled to  
6-(*tert*-butoxycarbonylamino)caproic acid using HATU to  
give VIII. The benzyloxycarbonyl protecting group was  
5 hydrogenated over Pd/C catalyst, and this free amine was  
then coupled to benzenesulfonyl chloride to give IX. The  
methyl ester was hydrolyzed with lithium hydroxide,  
followed by the deprotection of the *tert*-butoxycarbonyl  
protected 6-amino group using acidic conditions to give X.  
10 This amino acid was then coupled to methoxy-poly(ethylene  
glycol)-succinimidyl propionate (MW: 20,000) to give  
BIO-9257 (XI).

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BIO-9257

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### Example 8: OTHER COMPOUNDS

Bisphosphonates are now standard of care in myeloma treatment (see, e.g., Berenson et al., J Clin Oncol 16: 593-602 (1998)), and are effective at reducing bone lesions, but not tumor burden, in this model (see, e.g., Dallas et al., Blood 93: 1697-1706 (1999)). Since all myeloma patients are now on bisphosphonates, the efficacy of an VLA4 mAb or a small molecule, such as BIO8809, in combination with bisphosphonates will be tried.

Thalidomide is the latest drug with reported efficacy in myeloma patients (Desikian et al., Blood 98: 492-4 (2001)). Other drug candidates include erythropoietin, and mAb blockers of IL6 and IL15 (Mittelman et al., Proc Natl Acad Sci USA, 98: 5181-5186 (2001); Tinhofer et al., Blood 95: 610-618 (2000); Bataille et al., 1989). The efficacy of an VLA4 mAb or a small molecule, such as BIO8809, in combination with any one of these compounds, as well as with melphalan, could be tried.

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